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AFFERENT IMPULSES IN THE NERVES SUPPLYING THE URINARY BLADDER

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The object of the research described in the present communication was to study the nerve action potentials relating to the activity of the urinary bladder. Stella [1934] demonstrated the presence of stretch receptors in the frog's bladder and Evans has published an account of the afferent impulses [1936]. The present work was begun some years ago, it dealt with both afferent and efferent discharges, but this communication is restricted to a description of afferent discharges. As regards the efferent I find that my experiments have led me to conclusions which differ in several respects from those made by Evans. These points of difference are therefore subjected, at present, to further investigation.

Method

Dogs were used in all experiments. The experiments were made on spinal or decerebrate animals as well as on animals anæsthetized with chloralose, chloral hydrate or sodium luminal. It was soon found that anæsthesia did not modify the character of the afferent discharges described below. The bladder was exposed by an incision in the mid-ventral line. The afferent discharges were recorded from the peripheral end of the pelvic visceral nerve after section close to its origin from the sacral plexus. The branches innervating other pelvic viscera besides the bladder were cut. The hypogastric nerves were exposed by the same operation. They were tied and cut near the inferior mesenteric ganglion and dissected down to the hypogastric plexus. A number of experiments were performed on animals in which the pelvic visceral or the hypogastric nerves on one or both sides were cut and allowed to degenerate. Afferent discharges were then recorded from the fine long branches running along the

lateral wall of the bladder. This was necessary in order to prove that the impulses recorded were due to changes in the bladder itself and not to changes in any other neighbouring viscera. A number of experiments were also made on isolated bladders which were kept in warm oxygenated Tyrode's solution, the nerves of the bladder being carefully preserved.

Silver silver-chloride electrodes were used for leading off the action potentials which were recorded by a Matthews' oscillograph system. A cannula connected to a reservoir containing warm saline was introduced into the apex of the bladder. The diameter of the reservoir was about 10 cm. so as to allow as little change as possible in the filling pressure during the distension of the bladder. For recording the bladder volume the top of the reservoir was connected by means of a rigid tube to a volume recorder placed near the slot of the camera. The intravesical pressure was registered by a membrane manometer carrying a small Matthews' oscillograph concave mirror with a focal length of 5 m.

Afferent discharges in the pelvic visceral nerve

The general characteristics of the discharge in this nerve are more or less the same in different preparations. The differences concern only details and depend in the same preparation on the temperature, position and tone of the bladder.

Two types of discharge can be observed when the bladder is empty:

(1) Occasional impulses of the fast type due to the activity of a few nerve endings. The frequency of the discharge in single-nerve fibres is from 1–5 per sec. The impulses are more frequent when the tone of the bladder is increased, for instance, as a result of exposure.

(2) Fast impulses coinciding with each heart beat. These are usually due to more than one nerve ending. With each cardiac systole the nerve endings give up to ten impulses (Fig. 1 A). These impulses are probably due to nerve endings which lie near the vesical artery, and which are mechanically stimulated by the pulse pressure, for the impulses disappear if the artery is ligatured and sometimes if the position of the bladder is changed. Impulses of this kind were absent in spinal animals presumably on account of the low blood pressure, but the effect of changes of blood pressure upon this discharge was not studied. It is interesting to note that nerve endings responding to changes in pulse pressure have been described in mammalian muscle [Matthews, 1933], in the lung [Adrian, 1933] and in the mesentery [Gammon & Bronk, 1935].

If, after tying the urethra, the bladder is filled with warm saline through a cannula introduced into its apex, the following types of discharges are observed:

(1) At the moment the fluid begins to enter the bladder an outburst of impulses takes place. This discharge is not maintained however for a long time, and it greatly depends on the rate of filling of the bladder. For example, when the bladder is filled at a pressure of 5 cm. of water or less, the initial outburst is usually absent. It becomes more conspicuous and persistent with increase in the rate of filling as shown in Fig. 1 B, C.

Fig. 1. Afferent discharges in the pelvic nerve. A, synchronous with heart beat, bladder empty. B and C, initial outbursts of discharge produced on filling bladder at a pressure of 15 and 25 cm. H_2O respectively. This and all the other records are to be read from left to right.

(2) At the termination of the initial outburst the nerve either remains quiescent or sometimes shows occasional impulses until a certain intravesical pressure, which varies from one preparation to another, has been developed. The impulses then reappear at first in a few and then in more nerve fibres. If the filling of the bladder is now discontinued the discharge fades away in about 5 min. owing to the relaxation of the bladder wall. The discharge recurs if more fluid is admitted into the bladder so as to bring back the pressure to the value at which the discharge made its appearance. This pressure is approximately constant in each preparation. The discharge can also be elicited by application of external pressure to the wall of the distended bladder. During the fading of the discharge there is usually a stage at which the impulses can be seen to occur

synchronously with each respiratory movement. The increase in the intravesical pressure due to respiration is usually small, the bladder being exposed and outside the abdominal cavity. The increase is, however, sufficient to excite the nerve endings, as can be shown by eliciting a similar increase in the intravesical pressure by other means. When the bladder is filled beyond a certain volume, varying greatly in different preparations (100–350 c.c. in the dog), the high intravesical pressure as well as the discharge are maintained for a much longer time (20 min. or more). Frequently the activity of the end organs becomes intermittent, the different end organs tending to discharge at the same time. If the bladder is kept warm the pressure record shows slow waves each occupying from 30 to 60 sec.; the pressure rises and falls by 3–5 cm. of water. These slow waves are due to isometric contractions of the detrusor muscle. They are independent of the nerve supply to the bladder. The discharge in the pelvic nerve is greatly augmented by these contractions when they are present.

(3) In most experiments volleys of impulses were observed when the bladder was allowed to empty itself through the cannula. The discharge was found to depend to a great extent on the rate of emptying.

The effect of contraction of the bladder wall

The presence of afferent impulses in the pelvic visceral nerve during the automatic contractions of the bladder suggested that the nerve endings in the bladder wall might be stimulated either by the contraction of the detrusor muscle or by the increase in the intravesical pressure which accompanies these contractions. In order to study the mechanism by means of which the bladder contraction brings about an afferent discharge in the pelvic visceral nerve, use was made of the fact that the separate slender branches, running from the hypogastric plexus along the lateral ligament of the bladder, supply areas of the bladder which overlap each other to a very considerable extent. Each branch is made up of afferent and efferent fibres and, furthermore, it contains fibres from the pelvic visceral as well as from the hypogastric nerve. In order to avoid recording impulses of hypogastric origin, the right hypogastric nerve was cut in three dogs and allowed to degenerate. The animals were used for experiments on the tenth to fifteenth day after the operation. The bladder was exposed, cut longitudinally, and a large strip of it was attached to a light isometric spring lever carrying a mirror for optical registration of the contractions. The nerve branches supplying the strip were carefully preserved. The contraction of the strip was evoked by stimulation of a

pelvic visceral branch, while the afferent impulses were recorded from a neighbouring branch. Fig. 2 shows simultaneous records of the isometric contraction and of the afferent impulses produced by it. It can be seen that the impulses occur at the moment when the maximum tension is reached, and that on relaxation they disappear when the muscle tension is still above the resting level. The nerve was stimulated in this experiment by a faradic current with a frequency of 60 shocks per sec. When the maximum tension is maintained for a longer time by using stimuli of lower frequencies the discharge, although somewhat more prolonged, fails nevertheless fairly rapidly. The sensory end organs which are stimulated

Fig. 2. Afferent discharge in pelvic nerve. Upper tracing: isometric contraction of detrusor muscle. Lower tracing: nerve impulses.

by the contraction of the muscle must be different from those responding to distension of the bladder, since the former invariably show a much greater rate of adaptation than the latter.

Single nerve-fibre discharge

Following the method of Adrian & Bronk [1928], the pelvic visceral nerve was dissected until a regular discharge was obtained. In some experiments the dissection was carried out only in a small strand of the nerve trunk, the rest of the nerve being used for stimulation. Analysis of single nerve-fibre discharges shows that the nerve endings can be divided into two groups. The first most probably bears no relation to micturition and is situated outside the wall of the bladder; the second consists of endings incorporated in the bladder and stimulated by changes affecting its wall.

The nerve endings which are stimulated by the pulse pressure of the vesical artery, as well as those which are stimulated by changes in the

position of the bladder occurring during its filling, belong to the first group. At the beginning of filling of the bladder a number of nerve endings give about ten to fifteen impulses, after which the discharge completely disappears. This was found, however, to occur only when the bladder was kept in some definite position, which varied from one experiment to another. After changing the position of the bladder by turning it aside or suspending it vertically by the cannula, these discharges could not be observed any more. This type of discharge was found in about 25 p.c. of the experiments.

At least two types of nerve-ending discharges which depend on the changes in the bladder wall could be distinguished. They can be illustrated by the following two experiments:

Exp. 32. Male dog, 7 kg., sodium luminal 0·5 g. slowly injected intravenously. The pelvic visceral nerve is dissected to the stage of single nerve discharge. The bladder is connected by a cannula to a reservoir placed at a height of 20 cm. and containing warm saline; the urethra is ligatured. The bladder is filled by successive portions of 25 c.c. at a time, 5 min. intervals being made between each filling. At the beginning of each filling the nerve ending gives from fifteen to thirty impulses. At the start of each discharge its frequency rises to about 30 per sec., and then it regularly declines and finally stops in about 3 sec. At the fourth filling the discharge is found to be greatly diminished, which is due to the sudden decrease in the rate of the filling of the bladder. The time taken to introduce each successive 25 c.c. portion is 6, 6, 8·5 and 29 sec. respectively. After the diminution has taken place the original discharge can be reproduced on raising the reservoir to bring back the filling to its previous rate. At the beginning of the emptying of the bladder the discharge reappears, though fewer impulses are produced. The nerve ending gives from ten to twenty impulses. When the emptying is made in successive portions with intervals of 5 min. a discharge appears at the beginning of each emptying.

It was found that the same nerve endings were stimulated also by the contractions of the detrusor muscle, and in fact by any sudden change affecting the bladder wall. About 40 p.c. of the nerve endings examined behaved in this manner.

Exp. 41. Female dog, 6·5 kg., anæsthetized and operated as before. No discharge could be found, although the bladder was filled with 60 c.c. which was all that it would hold at a pressure of 10 cm. of water. When more fluid was introduced by raising the pressure by 20 cm. a discharge occurred. The maximum frequency of the discharge was found to increase with the intravesical pressure until at a pressure of 45 cm., the bladder volume being 200 c.c., the frequency of the discharge from the single end organ was about 300 per sec. At a constant bladder pressure the discharge declined in rate at first rapidly and then slowly, but even after 15 min. the end organ was still found to be active, although irregularly. Sometimes the activity of the organ became intermittent during the early decline of the discharge. Periods of rest alternated with periods of activity. During the periods of activity the frequency of the discharge was usually greater than during the time when the discharge was continuous (Fig. 3).

About 35 p.c. of the examined end organs behaved in this manner. The minimal intravesical pressure needed to excite these nerve endings greatly differs from one preparation to another, but it is approximately constant for each ending. The minimal pressure values found in six preparations were 10, 15, 8, 10, 20 and 25 cm. of water. These nerve endings were found to be unstimulated by the contractions of the detrusor muscle.

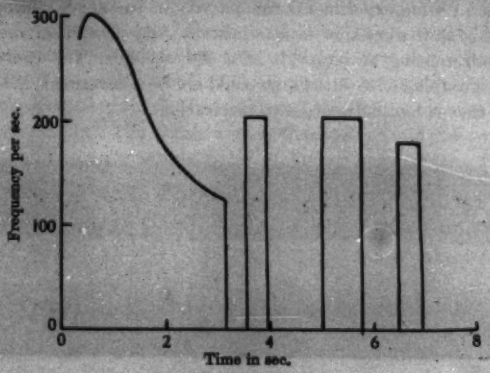

Fig. 3.

AFFERENT DISCHARGE IN THE HYPOGASTRIC NERVE

The first few experiments showed that the impulses recorded from the hypogastric nerve are very similar in character and time relations to those found in the pelvic visceral nerve. Langley & Anderson [1894] have shown that medullated fibres belonging to the pelvic visceral nerve may run in the hypogastric nerve as high up as the inferior mesenteric ganglion and then loop back to reach the bladder. This suggested that the similarity of the afferent discharges might be due to the presence of the pelvic visceral fibres in the hypogastric nerve. In order to avoid this source of error, all the experiments on the hypogastric nerves were made after degeneration of the pelvic visceral nerve on one side. The nerves were cut aseptically about 14 days before the experiment.

Occasionally single or groups of fast impulses could be recorded from the hypogastric nerve in these operated animals. These impulses bore no obvious relations to any activity of the bladder, and could equally be observed when the bladder was empty or full. Most probably they are

due to the activity of extravesical nerve endings and have nothing to do with micturition. When the bladder was filled with a large amount of fluid so as to cause considerable distension, slow waves of small magnitude made an appearance. This slow discharge shows a very slow adaptation; it continues as long as the high intravesical pressure is maintained. Fig. 4A shows an example of these slow waves recorded 25 min. after the bladder had been filled to an extent which increased the pressure to 70 cm. of water. In some experiments the activity of the nerve endings giving rise to this slow discharge is intermittent, the periods of rest and of activity alternating at intervals of a few seconds. The cause of this rhythmic grouping of the discharge could not be ascertained; it was found however that it had no relation to respiration.

Fig. 4. Afferent discharge in the hypogastric nerve. A, pelvic nerve cut 14 days before experiment: pressure 70 cm. water. B and C, from another preparation showing synchronous activity of nerve endings. Sensitivity 0·1 mV. = 7 mm. deflexion.

Occasionally the nerve endings giving rise to this slow discharge seem to act in unison. Fig. 4B shows two types of waves with frequencies of about 5 and of about 1 per sec. respectively. The time relations and the magnitude of each wave are identical, and one could have attributed this discharge to single nerve endings were it not for their much greater magnitude than is likely to occur in single sympathetic nerve fibres. It is probable that these waves are due to groups of nerve fibres acting simultaneously. The frequency of the waves increases with a rise of the intravesical pressure, but the synchronization is as well marked at a low (Fig. 4C) as well as at a relatively high frequency (Fig. 4B). It may be assumed either that the nerve endings have become connected in some way so that they act synchronously, or that they are exposed to a rhythmic and a steady stimulus. This effect appeared only in a limited number of experiments.

Impulses in relation to the urethra

For the study of the afferent discharge in the pudic nerve the anterior part of the pelvic girdle was removed. In the female the urethra was exposed in its full length and cleared from the fascia and the fat surrounding it. In the male only the intrapelvic portion of the urethra was used. The afferent discharges were recorded from the fine branches of the pudic nerve which run along the urethra and also from the pelvic visceral and the hypogastric nerves. The male and the female urethra presented no difference in its behaviour, but most experiments were made on females on account of the easier access to the organ. A cannula was introduced through an opening in the ventral surface of the bladder into the proximal part of the urethra. The cannula was connected with a reservoir and a manometer. A second cannula with a similar arrangement was connected with the distal part of the urethra. Warm saline could thus be made to pass into the urethra from either direction. By clamping the outlet the effect of distension without a simultaneous flow of saline could be studied. The resistance of the urethra to the flow of saline varied from one preparation to another, which probably depended on the depth of the anæsthesia. In deeply anæsthetized animals a pressure as low as 10 cm. of water was sufficient to overcome the urethral resistance.

The effect of distension

On examining the peripheral end of the pudic nerve there is usually found a constant faint discharge, even though the urethra and the bladder are not interfered with. On distending the urethra with pressures which are just sufficient to overcome its resistance there is either no change in the resting discharge or a transient augmentation of it lasting for a few tenths of a second. No persistent discharge was found until the distending pressure was increased to 50–70 cm. of water. Fig. 5A shows the discharge which accompanies a distension of the urethra with a pressure of 85 cm. of water. This discharge was maintained for about 10 min. after which it became intermittent (Fig. 5B). The resting periods gradually became longer, and after 20–30 min. the discharge disappeared. If the urethra is now emptied and the distension repeated 5 min. later the same sequence of events is reproduced. With shorter intervals the discharge is not so pronounced and not maintained.

Distension of the urethra evoked discharges also in the pelvic visceral and in the hypogastric nerves. The first shows a very rapid adaptation and occurs only during the process of distension, rapidly disappearing what-

ever the distending pressure may be. It was found in some experiments that the fibres carrying this discharge all pass on one side, showing a marked preference for the right nerve. The end organs responding to the distension of the urethra and sending their impulses along the hypogastric nerves need the highest pressure to excite them. A pressure of above 100 cm. of water was necessary to evoke this discharge. The nerve fibres carrying the discharge must be very few in number, as it is occasionally possible to detect what is apparently a single nerve-ending discharge from the hypogastric nerve without any preliminary dissection.

Fig. 5. Afferent impulses in pudic nerve due to distension of the urethra, pressure 85 cm. H_2O. A, beginning of discharge. B, 10 min. later.

The effect of flow of saline through the urethra

The flow of saline acts as a very effective stimulus to the sensory nerve endings of the urethra. The discharge in the pudic nerve is found to be maintained by the flow at pressures much lower than those needed to evoke a continuous discharge due to distension. The discharge begins together with the flow and builds up in 1–5 sec., after which it is maintained, without showing any appreciable change, as long as the fluid is passing through the urethra. When the flow is stopped the discharge takes 1–2 sec. to disappear completely. The direction of the flow of saline through the urethra is immaterial as far as this discharge is concerned. No discharge could be found in either the pelvic or the hypogastric nerve as a result of the flow, unless the flow was carried out at such a high pressure as to cause an appreciable distension of the urethra.

Discussion

Langley & Anderson [1894–95] demonstrated that one-tenth of the fibres in the hypogastric nerve and about one-third of those in the pelvic visceral nerve are afferent. The study of the afferent impulses from the bladder demonstrates that, of the two sets of nerve fibres, the pelvic visceral

nerves register changes in the intravesical pressure more faithfully than the hypogastric nerves. Investigation made upon the single nerve-fibre discharge of the pelvic visceral nerves shows the presence of nerve endings responding with frequencies which vary in the same direction as the changes in the intravesical pressure. The rate of adaptation of these nerve endings varies, some adapting rapidly while others adapt fairly slowly. With moderate distension of the bladder, all discharge usually disappears after an interval of about 10 min. There is a considerable difference in the thresholds of excitation of these nerve endings, some beginning to respond at a pressure of 8 cm. of water while others need a pressure as high as 25 cm. of water.

The urinary secretion observed in animals under the same experimental conditions was 4–20 c.c. per hour, which was less than the slowest rate of the artificial filling of the bladder evoking a discharge. Slow filling produces none or only a weak discharge, since the bladder musculature has time to adapt itself by lengthening so that the filling proceeds in almost isotonic conditions. Mosso & Pellacani [1882] were the first to point out that the stimulus exciting the desire to micturition is closely related to the intravesical pressure but not to the quantity of the vesical contents. The recording of the afferent discharges definitely confirms these observations. In the normal animal, therefore, the initial outburst of discharge described above does not occur unless the bladder is artificially filled for lavage or as a procedure preliminary to an operation.

At a certain stage of filling of the bladder the intravesical pressure begins to rise more rapidly. But in the normal animal the end organs may become excited before this stage is reached since the increase in the intravesical pressure due to respiratory movements, contractions of the abdominal muscles and contraction of the musculature of the bladder itself will determine the stimulus much earlier.

In the hypogastric nerve only the slow potential waves which are observed during considerable distension of the bladder and which show a very poor adaptation have to be considered. The majority of the fibres giving rise to fast impulses belong to the pelvic visceral nerves. The fast impulses recorded from the hypogastric nerve after a preliminary degeneration of the pelvic nerves have no relation to the intravesical pressure, and disappear after removal of the bladder outside the animal and after the tissue surrounding it is cleared away. The slow discharge remains under these conditions undisturbed. The poor adaptation of the slow waves in the hypogastric nerves, and the fact that they occur only during a great distension of the bladder, suggest that the waves might be responsible for

bladder pain. This conclusion is supported by the observation that in the decerebrate preparation the degree of bladder distension evoking this slow hypogastric discharge is accompanied by strong motor reflexes. It was also found that in anæsthetized animals severe distension of the bladder caused a rise in blood pressure from 15 to 30 mm. Hg. Cutting the pelvic visceral nerves had no effect on this pressor reflex, while cutting the hpogastric nerves completely abolished it in three preparations and greatly reduced it (to 5 mm. Hg) in two others. This negligible pressure response disappeared after the removal of the inferior mesenteric ganglion. If the assumption is justifiable that the impulses causing a rise in the blood pressure in anæsthetized preparations lead to a sensation of pain in the normal intact animal, then it becomes highly probable that the main function of the afferent fibres in the hypogastric nerve is to carry painful impulses to the central nervous system.

Summary

1. The activity of the sensory nerve endings in the bladder and the urethra has been investigated.

2. The sensory nerve endings related to the bladder can be classified into two main groups. The first group comprises endings situated outside the bladder wall and stimulated by changes in the position of the bladder or by the pulse pressure. These probably bear no relation to micturition. The second group comprises endings situated in the bladder wall. Some of these endings adapt rapidly, and are stimulated by rapid changes in the bladder contents and by the contraction of the detrusor muscle. Others adapt slowly and are stimulated by the rise of the intravesical pressure. These latter endings vary greatly in the threshold of their excitation. Above the threshold, the frequency of the discharge increases with the intravesical pressure.

3. Impulses resulting from stimulation of both groups of nerve endings pass through the hypogastric and the pelvic visceral nerves. The discharge in the hypogastric nerve is, however, mostly due to an admixture of fibres belonging to the pelvic visceral nerves.

4. Evidence is given to show that the main function of the afferent nerve fibres in the hypogastric is to carry painful impulses. These occur on maximal distension of the bladder and adapt very slowly.

5. On distension of the urethra impulses pass through the hypogastric pelvic visceral as well as the pudic nerves.

6. The flow of saline through the urethra evokes impulses only in the pudic nerves.

The subject of this work was suggested by Prof. G. V. Anrep, to whom I wish to express my warmest thanks for his constant advice and criticism throughout the investigation.

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OBSERVATIONS ON THE RENAL CIRCULATION AND SECRETION IN THE DOG, WITH SPECIAL REFERENCE TO THE EFFECT OF PITUITARY (POSTERIOR LOBE) EXTRACT[1]

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In the studies of Velden [1913] and Pick [1929, 1932], and in a recent paper by one of us [Samaan, 1935*d*], attention was drawn to the very high potency of post-pituitary extract in inhibiting the diuresis which follows the ingestion of water. From the investigations mentioned above, it was thought that information concerning the mode of action of various agents known to affect the rate of urinary flow might be obtained by investigating the changes in the renal circulation induced by these agents. The introduction of Rein's thermo-stromuhr [1928, 1929] for the study of blood flow through organs has solved one of the serious difficulties encountered in investigations on the intact animal. In fact the present work was made possible by using Rein's thermo-stromuhr, and the renal circulation was studied in both conscious and anæsthetized dogs.

Section I. Observations on conscious dogs

Methods

Twenty-one dogs of quiet disposition, weighing from 8 to 21 kg., were trained to receive a stomach tube without resentment. The "element" of Rein's apparatus was applied to the renal artery under ether narcosis, and the animals were then allowed to recover. In the first series of seventeen dogs observations on the renal circulation were made between the 4th and 16th hours after complete recovery from ether. In the second

[1] Preliminary reports: Handovsky & Samaan (1935), *J. Physiol.* **85**, 30 *P*; (1936), *C. R. Soc. Biol.*, Paris, **122**, 122.

series of four dogs the application of the "element" was made under strict surgical asepsis, and the study of the renal circulation was extended to the 6th day after the operation. The experimental findings in the two series were essentially the same.

Under ether narcosis the renal artery, commonly the left, was exposed extraperitoneally through a small lumbar incision. A short segment, free from branches, was then cleaned, care being taken to avoid the renal nerves, as far as possible, unless it was intended to denervate the kidney. After applying the cuff of the thermo-stromuhr "element" to the artery and securing it in position by a ligature or by celloidin-acetone solution, the ureter was then cannulated close to the pelvis of the kidney by inserting a multi-perforated silver cannula, which conducted the urine to the outside through a fine rubber tube. The wires of the "element" and the rubber tube were fixed to the ilio-costal muscle in a convenient position in order to avoid kinking the artery and obstructing the urinary path. The lumbar wound was finally closed, but room was left for the exit of the tube and the wires, which were then protected from undue manipulations by applying bandages and a specially designed "operation coat". The operation could be easily completed within 10–20 min., and recovery from ether was very rapid and uneventful. The lumbar route was preferred to the abdominal because it gave a direct extraperitoneal approach to the renal artery.

The original papers of Rein [1928, 1929, 1935] should be consulted for the details of the apparatus, technique and calibration of the "elements". The principle of Rein's method for determining the rate of blood flow through the unopened blood vessel consists in warming the latter at a given point and measuring the change in the temperature by means of a differential thermocouple, the thermojunctions of which are placed on the blood vessel equidistant proximal and distal to the site of warming, and a sensitive galvanometer. If the heat applied to the blood vessel is kept constant, the difference between the temperatures of the thermojunctions would depend on the rate of blood flow. In the present work we employed a moving-coil galvanometer, Zernicke Za (Kipp and Zonen); complete period = 1·3 sec.; internal resistance = 7 ohms; external resistance = 50 ohms; diameter of mirror = 8 mm.; deviation of 1 mm. at 1 metre distance = 4×10^{-7} volt or 7×10^{-9} ampere.

In most experiments we employed "elements" originally designed by Rein; later, however, a modification in the shape of the "element's" cuff was introduced which proved more convenient for chronic application to the blood vessels.

In all experiments the animal was placed in a Pavlov stand, the wires of the "element" correctly connected to the plugs of Rein's apparatus and the urine of the operated kidney collected and measured at intervals of 5 min. Not uncommonly kymographic records were taken of the urinary flow [Samaan, 1935*c*], general blood pressure from the cannulated brachial or carotid artery [cf. Samaan, 1935*a*] and of the respiratory movements. Of the seventeen dogs in the first series anuria was observed in four animals, which were therefore discarded.

Results

(1) *The renal blood flow during rest.*

The rate of blood flow through the renal artery in quiescent dogs was nearly constant, with small variations only. In two dogs, however, the renal blood flow showed slow periodic oscillations which assumed the form of fairly regular waves, and which were not related to cardiac or respiratory movements.

On the other hand, if the animal was restless or slightly disturbed a temporary but conspicuous reduction in the renal circulation was commonly observed (Fig. 1, II).

The average rates of blood flow through one kidney in the different dogs during rest lay within 1·73–3·80 c.c./g. kidney/min. The alterations in the renal circulation, produced in the same dogs by the agents dealt with later in this paper, varied between 0·9 and 6·4 c.c./g. kidney/min.

(2) *The renal blood flow during water diuresis.*

A preliminary "hydrating" dose of tap water (15–20 c.c. per kg. by stomach tube) was given, and the animal was returned to its kennel. After about 2 hours the observations on the renal circulation and secretion were begun, and some 30–40 min. later the "experimental" dose of water was administered by stomach tube (20–30 c.c. per kg.) and repeated at intervals of about 2·5 hours. In a few experiments water was administered either (*a*) by allowing the animal to lap it voluntarily, or (*b*) by intravenous infusion of tepid tap water at 5·0–7·0 c.c. per min. for 36 min. [cf. Rabinovitch, 1927; Klisiecki *et al.* 1933].

Shortly after the administration of water by stomach tube the renal blood flow began to increase and reached its maximum, about 30–70 p.c. above the basal level, in 20–40 min. The urinary flow also began to rise 10–20 min. after the administration of water and reached its maximal rate in 35–60 min. The lag between the maximal rates of blood and urine

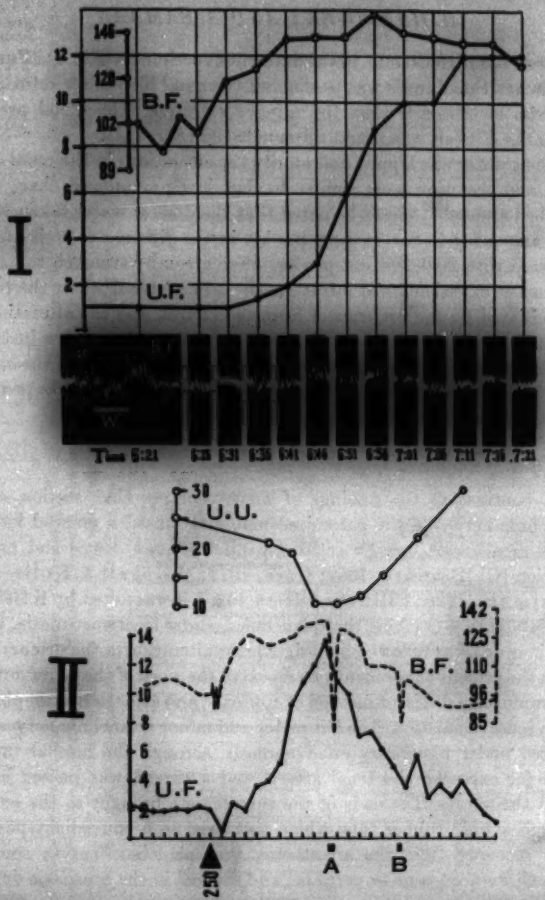

Fig. 1. The renal blood flow during water diuresis. I is taken during the first day after the operation; 14·0 kg. dog; weight of left kidney = 41·5 g. At *W*, 350 c.c. tepid tap water given by stomach tube. Successive tracings recorded every 5 min. II is taken from a 10·0 kg. dog, 4 days after the operation; weight of left kidney = 29·0 g. ▲ = 250 c.c. tepid water given by stomach tube. *A* = temporary restlessness. *B* = micturition of urine secreted by the unoperated kidney. *B.F.* = rate of blood flow through the left renal artery in c.c. per min. (Rein's thermo-stromuhr). *U.F.* = urine flow of left kidney in c.c. per 5 min. *B.P.* = blood-pressure in mm. Hg. *U.U.* = urea of urine in g. per litre. Abscissæ = time in 5 min.

flows was about 10–30 min. in the different experiments (Fig. 1). Furthermore, when the diuresis was passing off the renal blood flow returned to its initial level earlier than the urinary secretion. The blood pressure during the diuresis was practically unaltered (Fig. 1, I).

When water was lapped voluntarily the alterations in the renal circulation and secretion were similar to, but less pronounced than, those described above. It might be noted that the dose of water taken voluntarily amounted to 8·0–14·0 c.c. per kg. in the different experiments, as compared with 20·0–30·0 c.c. per kg. when given by stomach tube.

In most experiments of intravenously administered water the results were vitiated by the presence of hæmoglobinuria, and the alterations in the renal circulation and secretion were irregular. In the few instances in which hæmoglobin was absent from the urine until later in the experiment, the increase in the urinary flow was pronounced and was preceded by an augmentation of the renal blood flow.

(3) *Effect of unilateral splanchnotomy.*

In contrast to the findings of many workers that section of the splanchnic nerves in the narcotized mammal caused a marked increase in the renal blood flow [Burton-Opitz & Lucas, 1908] and urinary secretion [Cl. Bernard, 1859; Grek, 1912; Marshall & Kolls, 1919; Yoshimura, 1920; Ellinger & Hirt, 1925], it was noted by Klisiecki *et al.* [1933] that crushing the splanchnics, under local anæsthesia, in the conscious dog was followed by insignificant alteration in the urinary flow.

In the present experiments we repeated the work of the latter authors, but employed a different method of approach and also studied the changes in the renal circulation. The left major and minor splanchnic nerves were exposed under temporary ether narcosis, through the lumbar incision made for exposing the renal artery, and a thread was passed loosely round the nerves. The ends of the thread were brought to the exterior through a wide rubber tube which was fixed in a convenient position. After recovery from the anæsthetic, the splanchnic nerves could be blocked, by novocaine or percaine, and severed in the conscious dog, the thread acting as a guide to their site.

Fig. 2 shows the results of a typical experiment. At *A*, functional block of the splanchnics was attempted by 2·0 p.c. percaine applied on cotton wool through the rubber tube. A gradual increase in the renal blood flow was observed. At *B*, the nerves were cut with a pair of scissors, passed down the rubber tube, and the section was verified post-mortem. The immediate effects of cutting the nerves was a temporary

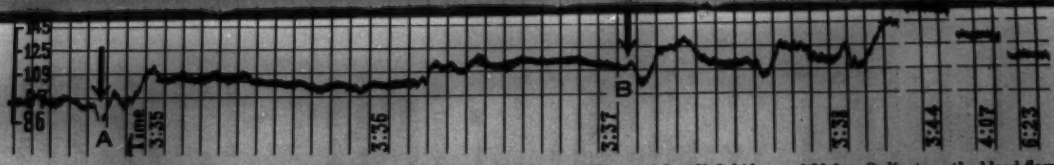

Fig. 2. The renal blood flow after homolateral splanchnotomy. Conscious dog, 9·0 kg.; weight of left kidney = ? 30·0 g. Ordinates = the blood flow through the left renal artery in c.c. per min. (Rein's thermo-stromuhr). Abcissæ = time in 5 sec. At *A*: cotton wool soaked in 2 p.c. percaine was applied to the left major and minor splanchnic nerves. At *B*: the same nerves were cut with scissors. Further details in text. The figures given at the foot of this and of the following tracings indicate the actual time of their registration. All tracings are to be read from left to right.

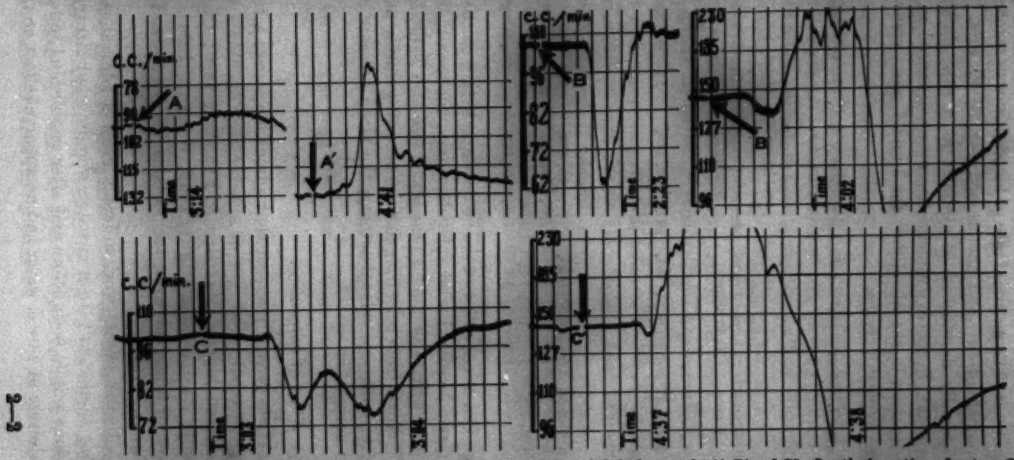

Fig. 3. Effect of adrenaline on the renal circulation in conscious dogs (*A*, *B* and *C*) before, and (*A'*, *B'* and *C'*) after the ingestion of water. Ordinates: blood flow in the left renal artery in c.c. per min. Abcissæ: time in 5 sec. Arrows: intravenous injection of adrenaline. At *A* and at *A'*, which are taken from a conscious bitch of 10·0 kg.: 0·0008 mg. adrenaline. At *B*, *B'*, *C* and *C'*, which are taken from another bitch of 11·5 kg.: 0·0045, 0·0045, 0·075 and 0·075 mg. adrenaline, respectively. *N.B.* The direction of the current in the galvanometer was the reverse in the two experiments of Fig. 3, hence the opposite deflections in the two experiments indicate the same changes in the blood flow.

irregularity of the renal blood flow which was succeeded by a progressive increase. The maximal rate of blood flow was reached in 2–10 min. after splanchnotomy and maintained for 20–50 min. Later, however, it gradually diminished to stabilize at a rate slightly higher than the original. In two experiments nerve section caused an immediate transient reduction in the blood flow for 3 and 7 min., respectively, followed by the typical increase.

TABLE I. Effect of unilateral splanchnotomy. Dog, 10·0 kg.; weight of left kidney, 31·0 g. Operation completed at 9.35 a.m.

Time p.m.	Urine flow in c.c. per 5 min. (left kidney)	Average minute renal blood flow in c.c., recorded every 5 min.	Average blood pressure in mm. Hg	Remarks
12.50	—	—	—	200 c.c. "hydrating" dose of tap water by stomach tube
3.00–3.25	0·75, 0·65, 0·6, 0·6, 0·6	118, 103, 110, 110, 103	115	Dog calm
3.26	—	—	—	2 p.c. novocaine applied to left splanchnic nerves
3.30	—	Irregular	Irregular	Splanchnics divided
3.25–3.55	0·55, 0·7, 0·95, 1·4, 1·4, 1·4	?, 95, 132, 138, 130, 130	112	—
3.55–4.25	1·4, 1·35, 1·2, 1·3, 1·3, 1·2	132, 123, 123, 123, 123, 123	110	—

The urinary flow of the homolateral kidney in the resting animal was clearly increased after splanchnotomy, the maximal rate amounting to 1·5–4·0 times as much as the initial (see also Table I).

Apart from brief irregularities occurring during and for a few minutes after unilateral splanchnotomy, the blood pressure returned practically to normal.

(4) *Effect of adrenaline.*

It is generally agreed that adrenaline causes a temporary reduction in the renal blood flow of anæsthetized mammals and of the isolated perfused kidney [Bardier & Frenkel, 1899; Cushny & Lambie, 1921; Richards & Plant, 1922; Winton, 1931]. The effects on the flow of urine, however, are variable [cf. Winton, 1931; Theobald & Verney, 1935*a*].

In the present work different doses of adrenaline (Parke, Davis and Co.), diluted to 1·0 c.c. in water, were injected intravenously in the conscious animal both before and after establishing water diuresis. Control injection of 1·0 c.c. water had no apparent effect on the kidney function.

Small doses of adrenaline, up to 0·0005 mg. per kg., caused a significant reduction in the renal blood flow which passed off in 0·5–1·0 min. (Fig. 3, *A* and *B*). Larger doses, averaging 0·001–0·01 mg. per kg., however, elicited peculiar changes in the blood flow through the kidney: (*a*) an initial brief reduction, followed by (*b*) a transient increase towards or above the original level—an increase which seemed to coincide with the period of maximal rise of blood pressure, then (*c*) a secondary reduction which was sometimes very pronounced, and finally (*d*) a return to the pre-injection level, or even to a higher rate which, however, subsided soon. The successive alterations in the renal circulation just described usually passed off within 2–6 min. (Fig. 3, *C* and *C'*; see also *B'*).

The urinary effects of adrenaline seemed to depend on the dose given. With small doses, for example, a very brief reduction was noted in most instances, while in others no effect or even a slight augmentation occurred. With the larger doses, on the other hand, an initial arrest of urinary flow for 1–4 min. was noted, and was followed by rapid recovery to the original rate in 10–20 min. (Table II). At this stage the urine contained albumin.

TABLE II. Effect of adrenaline. Dog, 16 kg.; weight of left kidney, 55·0 g. The "element" of Rein's thermo-stromuhr was aseptically applied to the left renal artery 2 days previous to the experiment.

Time p.m.	Urine flow in c.c. per 5 min. (left kidney)	Average minute renal blood flow in c.c., recorded every 5 min.	Average blood pressure in mm. Hg	Remarks
2.30	—	—	—	250 c.c. "hydrating" dose of tap water
4.55	—	—	—	400 c.c. water, given by stomach tube
4.55–5.35	0·7, 0·7, 0·9, 2·5, 3·5, 5·7, 7·4, 12·2	122, 165, 178, 190, 190, 182, 182, 190	130	Dog calm
5.35	—	—	—	0·1 mg. adrenaline in 1·0 c.c. water was injected intravenously
5.35–5.40	3·5 (1·8, 0·1, 0·2, 0·6 and 0·8)	(205, 78, 162, 211 and 195)	(185, 135, 118, 120 and 120)	The figures in brackets indicate average values every minute
5.40–6.15	6·2, 8·3, 12·5, 17·3, 16·1, 13·5, 10·0	195, 210, 210, 182, 160, 160, 153	125	—

The general blood pressure rose sharply, increasing after the larger doses of adrenaline by about 80–110 mm. Hg above the initial level, and subsided in 3 min. [cf. Samaan, 1935*b*]. Bradycardia was pronounced during the period of raised blood pressure.

It was frequently observed in any one animal that the effects of adrenaline on the renal blood flow became more pronounced after establishing water diuresis. For example, a small dose of adrenaline, which elicited pure transient reduction in the renal circulation during rest, produced the successive alterations in the blood flow which were commonly seen only after the larger doses, when injected in the same animal during water diuresis (Fig. 3, *B* and *B'*).

(5) *Effect of post-pituitary extract.*

Janssen & Rein [1928], working on decerebrate dogs, found that post-pituitary extract inhibited the urinary secretion without altering the rate of the blood flow through the kidney. Brings & Molitor [1931] noted an initial reduction in the kidney volume which passed off long before the antidiuretic action of the extract. Geiling *et al.* [1934] observed a transient diminution in the renal blood flow, followed by a prolonged increase.

The experiments described below and in Section II were undertaken to determine (*a*) the influence of different doses of post-pituitary extract on the renal blood flow, and (*b*) whether the "antidiuretic" action of the extract in the unanæsthetized dog during water diuresis, and the "diuretic" effect which is commonly observed under anæsthesia during saline diuresis, were related to differences in the behaviour of the renal circulation.

Post-pituitary extracts employed were the commercial preparations "Infundin" (Burroughs, Wellcome and Co.), "Pituglandol" (Hoffmann-Laroche and Co.), "Pitressin" (the pressor principle of post-pituitary extract, Parke, Davis and Co.) and "Posthypophyne" (Palfijn). The desired dilution of the extract with water was prepared immediately before use and 1·0 c.c. of the diluted fluid was injected intravenously, the dose being expressed in terms of the original extract. Control injections of 1·0 c.c. water had no effect.

During water diuresis small doses of post-pituitary extract, averaging 0·00001–0·00015 c.c. per kg., caused negligible or no decrease in the renal blood flow, but elicited a decided reduction of 10–20 p.c. in the urinary flow; the latter returned to its initial level in 10–25 min. (Fig. 5, *A*). The blood pressure was hardly altered. Larger doses of the extract, averaging 0·0004–0·05 c.c. per kg., produced an intense reduction in both the renal blood flow and urinary secretion. The blood flow fell steeply to as low as 22–54 p.c. the pre-injection level, but rapidly returned to normal in 1–9 min. (Fig. 5, *B*). With the large doses of the range just mentioned

the blood flow, after returning to its pre-injection level, increased progressively to reach rates about 1·5–2·0 times the original flow (Fig. 5, *C*). In contrast to the very short period of reduction in the renal circulation, the diminution in the urinary secretion lasted for 25–65 min., i.e., the urinary inhibition persisted not only during the period of reduction in

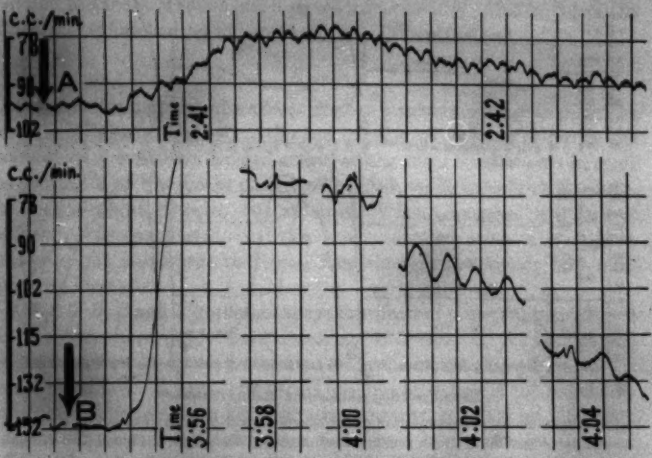

Fig. 4. Effect of post-pituitary extract on the renal circulation of a conscious bitch (*A*) before, and (*B*) after the ingestion of water. 10·0 kg. bitch; weight of left kidney = 29·0 g. Ordinates = the renal blood flow in c.c. per 5 min. Abscissæ = time in 5 sec. At arrow ↓: 0·1 c.c. "Infundin" intravenously. Note the intensified effect of the extract on the renal circulation during water diuresis.

the blood flow, but also after the return of the blood flow to or above that of the normal "water-diuresis" level (Fig. 5, *C*). The blood pressure rose after the administration of the extract, and subsided in 5–40 min.; the maximal increase varied between 20 and 60 mm. Hg and, in a few instances, was interrupted by a steep fall for a very brief period shortly after the administration of the extract.

The effects of post-pituitary extract on the renal function were also studied in resting animals previous to the administration of water. The alterations in the renal circulation were similar to, but less pronounced than, those observed after establishing water diuresis. Moreover, the

"antidiuretic" action of the extract was absent or greatly reduced and, not uncommonly, was followed by slight augmentation of the urinary flow.

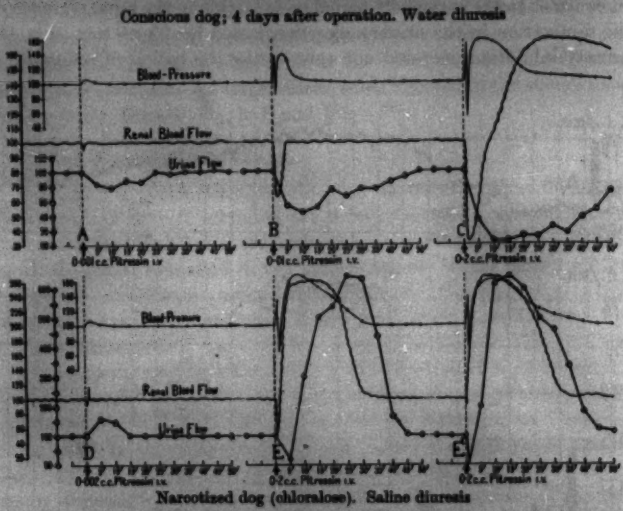

Fig. 5. Diagrammatic representation of the effects of post-pituitary extract on conscious and narcotized dogs. Dots: general blood pressure. Wavy line: the blood flow through the left renal artery (Rein's thermo-stromuhr). Circles: urine flow of the left kidney. Ordinates: the relative alterations in percentage of those of the normal "water-diuresis" level, which is regarded as 100 in all the cases. Abscissæ: time in 5 min. Arrow ↓: intravenous injection of post-pituitary extract. At *A*, *B* and *C*, which are taken from a conscious dog (12·0 kg.) during water diuresis: 0·001, 0·01 and 0·2 c.c. "Pitressin", respectively. At *D*, *E* and *E'*, which are taken from narcotized dog (10·0 kg.) during saline diuresis: 0·002, 0·2 and 0·2 c.c. "Pitressin", respectively.

Section II. Observations on anæsthetized dogs

The object of the following experiments was to determine whether the behaviour of the renal circulation during the "diuretic" effect of post-pituitary extract was different from that observed during the "antidiuretic" action described above.

Methods

After anæsthetization by chloralose (0·08–0·1 g. per kg. intravenously), eight dogs were given isotonic saline at body temperature by continuous infusion into the metatarsal vein at 0·12–0·3 c.c. per kg. per min. in the

different experiments. These experimental conditions seemed to favour a "diuretic" response to post-pituitary extract [cf. Craig, 1925; Knowlton *et al.* 1927; Macdonald, 1933]. The methods for studying the renal circulation, urinary secretion, blood pressure and respiration were the same as described in Section I. The commercial preparations "Infundin" and "Pitressin" were commonly employed, the required dose being diluted to 1·0 c.c. in saline and injected intravenously. Control injections of 1·0 c.c. saline had no apparent effect.

Results

Before dealing with the effects of post-pituitary extract it might be mentioned that the slow continuous infusion of isotonic saline in the narcotized dog had no decided action on the renal circulation. In most experiments no significant change was noted, while in only three observations a slight increase was recorded. When, however, the rate of infusion was augmented, or when a concentrated solution of sodium chloride was employed, the blood flow through the kidney was temporarily increased.

Fig. 5, *D*, *E* and *E'* represent the renal effects of post-pituitary extract in the narcotized dog. Small doses, up to 0·0002 c.c. per kg., caused slight transient increase in the urinary flow, but variable effects on the renal blood flow. A very brief period of slight reduction in the blood flow was not uncommon; in a few instances, however, no apparent alteration was noted, or only a brief reduction followed by a transient increase. The changes in the renal circulation were within 10–15 p.c. of the pre-injection rate, and usually passed off within 2–5 min. On the other hand, the augmentation of the urinary flow reached 30–70 p.c. above the original and disappeared in 10–15 min. Larger doses of the extract, averaging 0·002–0·005 c.c. per kg. intravenously, affected the renal circulation in the same way as in the conscious animal, namely, an initial steep reduction for 1–3 min. followed by rapid return to, or above, the normal. The delayed post-inhibitory increase of the blood flow usually followed the larger doses of the range just mentioned, and subsided in 20–40 min. (Fig. 5, *E*); in two observations, however, the initial phase of reduction in the blood flow was almost absent (Fig. 5, *E'*). The urinary secretion was markedly increased, reaching 3–8 times the original rate and lasting for 30–55 min., but was usually preceded by a very brief period of 1·0–3·5 min. of anuria or of marked inhibition.

The changes in the blood pressure were similar to those described in the conscious animal.

Discussion

(*a*) *Water diuresis.* It is clear from Fig. 1, I that the increase in the blood flow through the kidney during water diuresis cannot be ascribed to concomitant rise in the arterial blood pressure. The pulse pressure, however, was not determined, but it appears from the recent researches of Theobald & Verney [1935*b*] and Theobald [1936] that the relation of the urinary flow to pulse pressure and posture is not so important as was previously thought [cf. Hooker, 1907; White *et al.* 1926]. Further, the augmentation of the renal circulation is not dependent on the integrity of the renal nerves since it was observed in the chronically denervated kidney during water diuresis. It is possible, therefore, that the changes in the calibre of the renal vessels were related to alterations in the composition of the blood produced by the ingestion of water [cf. Handovsky, 1931, 1933]. In support of this view it may be mentioned that during water diuresis Priestley [1916] detected a slight reduction in the electrical conductivity of the serum, while Baldes & Smirk [1934] found a definite fall in the osmotic pressure of the blood, as determined by the vapour-pressure method [Hill, 1930]. In further agreement with the present observations on the renal circulation are the experiments of Jarisch & Liljestrand [1927], Grollman [1932] and Lequime & Denolin [1936], who found that the cardiac output was greatly increased after the ingestion of water.

(*b*) *Unilateral splanchnotomy.* The increase in the renal blood flow which followed homolateral splanchnotomy was most marked during the first hour, and was sometimes preceded by a very brief period of reduction. It is probable that the increase was dependent on the removal of renal vaso-constrictor impulses, but it is rather difficult to ascertain whether the occasional initial diminution in the blood flow was due to a transient mechanical stimulation of the peripheral stumps of the divided nerves, liberation of adrenaline [Burton-Opitz, 1916] or to interruption of renal vaso-dilator nerves [cf. Bradford, 1889].

The polyuria following splanchnotomy might be ascribed to the local circulatory changes in the kidney, and according to Peters [1935] it is due not only to increased glomerular filtration, but also to diminished activity of the renal tubules in reabsorbing water from their lumina.

(*c*) *Adrenaline.* In confirmation of previous researches [Sollmann & Hatcher, 1908; Richards & Plant, 1922; Winton, 1931; Unna, 1935] the principal action of adrenaline is a reduction in the renal circulation due to local vaso-constriction. The brief phase of augmentation

observed after large doses, and interrupting the period of diminution, is probably related to the steep rise in the general blood pressure which tends to force the blood through the constricted renal vessels (Fig. 3, *B'*, *C* and *C'*).

The urinary effects were studied in detail by Richards & Plant [1922] and Winton [1931]. According to these authors minute doses of adrenaline constrict the vasa efferentia of the glomeruli, leading therefore to a reduction in the renal blood flow, a rise in the glomerular blood pressure and increased glomerular filtration, and an augmentation of the urinary flow. On the other hand, larger doses of adrenaline constrict the afferent and efferent glomerular vessels so much that the renal blood flow is greatly reduced, and the glomerular filtration and urinary secretion are diminished. The latter effect of urinary inhibition, or arrest for a very brief period, was commonly observed in the present work and was found to pass off within 10–20 min. [cf. Theobald & Verney, 1935*a*].

(*d*) *Post-pituitary extract.* The experiments recorded above show that the changes in the renal circulation, produced by administering the extract to both conscious and anæsthetized dogs, were not unlike irrespective of whether the urinary responses were "antidiuretic" or "diuretic". These results indicated that the antagonistic urinary responses just mentioned were not dependent on differences in the behaviour of the renal circulation.

A close examination of the effects of post-pituitary extract during water diuresis seemed to point out that the "antidiuretic" action, described in Section I, was independent of the circulatory changes in the kidney. In fact, direct relationship did not exist between the rates of blood flow and urinary secretion; for example, small doses of the extract might cause no apparent change in the renal blood flow, but distinctly reduced the flow of urine. It might be argued, however, that the extract could reduce the flow of urine by local redistribution of blood in the kidney without causing any apparent alteration in the rate of blood flow through the renal artery. If one assumed that small doses of the extract constricted the vasa afferentia of the glomeruli and caused a reciprocal dilatation of the vasa efferentia, the renal blood flow might be unaltered although the glomerular blood pressure and glomerular filtration would become greatly reduced. This assumption, however, is not supported by experimental evidence [Richards & Plant, 1922; Burgess *et al.* 1933].

Again, large doses produced an initial, brief but steep, reduction in the blood flow followed by prolonged increase; in spite of this delayed

increase in the blood flow the inhibition of water diuresis persisted (Fig. 5, *C*). It might be argued that the initial intense ischæmia of the kidney, produced by the extract, could be responsible for a prolonged after-effect of urinary inhibition. This view is more fully dealt with in a further communication by one of us (Samaan), who found that partial or total anæmia of the kidney, produced by mechanical means, cannot explain the prolonged "antidiuretic" effect of post-pituitary extract.

The present observations on the inhibition of water diuresis by the extract appear to support the views of Burgess *et al.* [1933], who believed that the "antidiuretic" effect in mammals was due to stimulation of the process of water reabsorption by the renal tubules.

In contrast to the "antidiuretic" action of the extract on water diuresis in conscious dogs, the urinary effects of large doses in anæsthetized animals during saline diuresis seemed to follow the alterations in the renal circulation (Fig. 5, *E*). These results confirm Knowlton & Silverman [1918], Cushny & Lambie [1921] and McFarlane [1926]. With small doses, on the other hand, reduction in the renal blood flow was not uncommon although the urine secretion was augmented; it is probable that constriction of the vasa efferentia took place, leading to an increase in the glomerular blood pressure and in the glomerular filtration [cf. Richards & Plant, 1922; Richards & Schmidt, 1924].

Consideration of the factors determining the "diuretic" or "antidiuretic" effects of post-pituitary extract will be devoted to a further communication.

(*c*) *Relationships between blood pressure, renal blood flow and urinary secretion.* The observations recorded in this paper clearly indicate that parallel changes were not always observed in the blood pressure, renal blood flow and urinary secretion. For example, during water diuresis and after unilateral splanchnotomy the blood pressure was unaltered although the renal blood and urinary flows were increased. Again, with adrenaline and post-pituitary extract the blood pressure was increased, but the renal circulation was commonly reduced. During water diuresis, moreover, post-pituitary extract caused a divergence between the behaviour of the renal circulation and urinary secretion (Fig. 5, *A* and *C*).

Summary

1. The effects of various procedures on (*a*) the rate of blood flow through the renal artery, as determined by Rein's thermo-stromuhr, (*b*) the urinary secretion of the homolateral kidney, and (*c*) the

blood pressure, have been studied in conscious and in anæsthetized dogs.

2. The renal blood flow was found to be constant within narrow limits in resting dogs, but was reduced for a brief period when the animal was slightly disturbed.

3. It was found in conscious dogs that:

(*a*) The diuresis produced by administration of water was preceded by an increase in the renal circulation; the augmentation of the latter was independent of the renal nerves and was not related to a rise in the general blood pressure.

(*b*) Unilateral splanchnotomy was followed, either immediately or after a brief period of reduction, by an increase in the blood flow through the homolateral kidney and in the urinary secretion.

(*c*) Adrenaline caused a decrease in the renal blood flow which varied with the dose given; but the decrease was interrupted by a transitory augmentation if the concomitant rise in the blood pressure was great. The secretion of urine was commonly reduced for a brief period, and returned to its original rate within 10–20 min. After ingestion of water the effects of adrenaline on the kidney became more pronounced than before.

4. Small doses of post-pituitary extract caused in conscious dogs under water diuresis a reduction in the flow of urine, but an increase in the same in anæsthetized animals under saline diuresis; in both normal and narcotized dogs, on the other hand, the renal blood flow was practically constant or insignificantly reduced, while the blood pressure was hardly changed. Large doses produced marked "antidiuretic" or "diuretic" effects in conscious or narcotized dogs, respectively, but the renal blood flow in both cases showed an initial reduction followed by prolonged increase. The blood pressure increased after the injection of the extract, and usually subsided within 40 min.

5. The relation of the above findings to the kidney function has been briefly discussed.

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THE EFFECT OF ELECTROTONUS ON THE EXCITABILITY OF NERVE

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The decrease in threshold of nerve excitation due to catelectrotonus, which with strong electrotonus may become an increase in threshold, and the increase in threshold with anelectrotonus are well established. Less, however, is known of the effect of electrotonus on the time-factor of excitation. Cardot [1914] and Cardot & Laugier [1914*a*; see also Laugier, 1921] found that the intensity-duration curve is raised or lowered parallel to itself by electrotonus. This involves a change in chronaxie. On the other hand Kodera [1928] found that the threshold intensities for different durations are all multiplied by a certain factor in electrotonus. This means that chronaxie is not affected by electrotonus. Bouman [1931] found a change in chronaxie by electrotonus which could easily be removed by a small alternating current. Complete intensity-duration curves were not determined in his experiments. Little moreover is known about the influence of various ions on electrotonus. These points are considered in this paper, while the last part of it deals with the influence of inter-electrode distance on the effects of electrotonus.

Method

All experiments are performed on sciatic nerves of Hungarian *Rana esculenta*. The electrical circuit is essentially that used by Hill [1936*b*] for determining the time-factor k of excitation. The nerve was stimulated by repetitive condenser discharges at a frequency of 20 per sec. The nerve impulse was detected by its action current, which was integrated by a sensitive galvanometer (Kipp Zd). Semimaximal response was always taken in order to give constant results and avoid double intensity-duration curves [Hill, 1936*b*, p. 449]. Stimulating and action current electrodes were calomel half cells with sharp-edged wooden electrodes.

[1] Fellow of the Rockefeller Foundation.

The discharge time of the stimulating circuit is determined by a resistance R_1 of 1000 ohms together with the capacity C of the stimulating condenser. A resistance R_2 of 200,000 ohms is connected in series with the nerve, so that changes in nerve resistance could have no significant influence on the discharge time of the condenser. A calibrated slide-wire potentiometer is also connected in series with the nerve for the adjustment of the polarizing current. The total resistance of this instrument (Pye, Cambridge) is 30 ohms, so that changes in the setting of the contact did not appreciably change the resistance of the nerve circuit. The 200,000 ohms also make the polarizing current approximately independent of nerve resistance. The voltage of the polarizing battery was measured before and after each experiment and the intensity of the polarizing current adjusted by the potentiometer.

All nerves were soaked in oxygenated Ringer's solution before starting any experiment. For those experiments requiring soaking times of 10 hours and more the major part of the soaking was done in a refrigerator without bubbling oxygen. The nerve was removed from the refrigerator 2–3 hours before the actual experiments and placed in a freshly oxygenated solution at room temperature.

The experiments require considerable care in order to obtain constant results. Fortunately Hill's theoretical equation [Hill, 1936*b*] provides a check on the results obtained. A few experiments had to be discarded because they did not fit this equation.

The actual experiments were performed in the following way. A certain discharge time RC was taken. First the threshold was determined without electrotonus. Then catelectrotonus was switched on and after a few seconds the threshold was determined again. Then electrotonus was removed and after a short interval the threshold was determined again. Next, anelectrotonus was introduced and another determination of the threshold was made. Finally, after switching off the electrotonus the threshold was determined again. This procedure was repeated for different condenser settings and in most experiments also for constant current pulses of long enough duration to give the rheobase. Occasionally the entire experiment was repeated for different strengths of polarizing currents. In most experiments, however, it was thought advisable not to perform too many series of experiments on the same nerve muscle preparation, if constant and reliable results were to be obtained.

The constancy of the results was easily checked, as the threshold without electrotonus should be the same before and after each series of determinations. The threshold without electrotonus determined between

catelectrotonus and anelectrotonus was usually slightly higher than normal, due to the after-effect of catelectrotonus. The successive application however of catelectrotonus and anelectrotonus appears to give hardly any after-effect at all. At the end of each group of experiments with a certain strength of electrotonus the threshold was again determined with the same stimulus as at the beginning, to make sure that no appreciable change in threshold had taken place. All data had to fit Hill's equation for condenser discharges. From this the factor k was determined.

Results

Part I. *The influence of electrotonus on the intensity-duration (E–RC) curve*

Provided that the precautions mentioned above are taken, reliable results are obtained. Cardot & Laugier's statement that electrotonus simply shifts the intensity-duration curve up and down could not be confirmed. It is obvious from Table I that the difference between the

Table I. Thresholds (volts) for stimuli of long and short duration, with and without electrotonus

Exp. no.	Polarizing current μA.	Without electrotonus		Catelectrotonus		Anelectrotonus	
		Long	Short	Long	Short	Long	Short
1	0·5	1·06	7·4	0·9	6·55	—	—
2	1	1·33	10·0	0·96	8·9	1·91	13·3
3	2	1·46	22·6	0·92	15·6	2·73	34·6
4	5	1·44	15·2	0·68	8·7	4·46	33
5	1	1·36	25·4	1·04	21	1·89	32·4
6	2	1·16	20·4	0·62	13·6	2·34	31·9
7	5	2·51	16·4	1·26	9·5	7·2	36
8	1	1·62	31·2	1·24	26·2	2·30	36
9	2	1·28	21·4	0·92	18·2	2·13	25

thresholds for long and short duration is not independent of electrotonus as it would be if their conclusions were correct.

It was impossible also to confirm Kodera's statement that the ordinates of the intensity-duration curve with electrotonus bear a constant ratio to those without electrotonus, involving no change in time-factor of nerve excitation. On the contrary a clear change in the time-factor k was always found. Table II gives the time-factor k calculated from the E–RC curves for the same experiments as are given in Table I. It is found that the factor k is increased in catelectrotonus and decreased in anelectrotonus. In determinations of the U–V curve [Hill, 1936*a*] by short test shocks it is found that the curve does not return to its original level as a result of electrotonus. The amount of this deviation

TABLE II. The effect of electrotonus on the time-factor k of normal nerve

Exp. no.	Polarizing current μA.	Factor k: msec.		
		Normal	Catelectrotonus	Anelectrotonus
1	0·5	0·28	0·50	—
2	1	0·48	0·63	0·35
3	2	0·29	0·34	0·16
4	5	0·31	0·45	0·19
5	1	0·30	0·40	0·40
6	2	0·25	0·35	0·17
7	5	0·39	0·44	0·33
8	1	0·21	0·44	0·13
9	2	0·71	1·00	0·39

is obviously the result of two counteracting effects: firstly, the reduction in threshold due to catelectrotonus and secondly, the increase in k (and therefore a relative increase in threshold for short stimuli) in catelectrotonus.

Part II. *The influence of different ions on the effects of electrotonus*

The double effect of electrotonus on threshold and time-factor offered an opportunity to study the effect of different ions. The intensity-duration curve can be taken with and without electrotonus at the same time. This avoids the comparison of results obtained on the same preparation before and after intoxication, and therefore eliminates any experimental uncertainties, allowing also for long soaking times if the condition of the nerve after prolonged soaking permits reliable data to be obtained.

The effect of potassium ions. After preliminary soaking in normal Ringer's solution the nerves were soaked in a solution containing excess potassium (ten times normal in most experiments). An electrotonus experiment was then performed in the usual way. A soaking time of 2 hours was found to be necessary, longer soaking times did not change the results.

Table III gives the changes in threshold with electrotonus in a nerve which has been treated with excess potassium. The results confirm those of Chweitzer [1935] so far as catelectrotonus is concerned: catelectrotonus instead of lowering the threshold, as it does in normal nerve, raises it in the potassium-soaked nerve. According to Chweitzer the effect of anelectrotonus is not reversed after the potassium treatment. In some of my experiments the effect of anelectrotonus also was reversed. The effect, however, of anelectrotonus is not as constant as that of catelectrotonus, and for no obvious reason the reversal in anelectrotonus does not always take place.

TABLE III. The effect of electrotonus on the threshold for long-lasting currents in a nerve soaked in Ringer's solution containing excess potassium

Exp. no.	Polarizing current μA.	Threshold: volts		
		Normal	Catelectrotonus	Anelectrotonus
1	1	4·5	5·0	4·4
2	0·5	4·7	4·9	4·4
3	5	1·5	1·8	1·8
4	5	1·7	2·0	—
5	5	1·3	1·6	1·5
6	5	1·4	1·9	—

As the soaking in ten times normal potassium reverses the effect of catelectrotonus, it is obvious that there must be some concentration which just abolishes the effect of catelectrotonus. This has a certain importance, since electrotonus provides a disturbing factor in the determination of the U–V curve [Hill, 1936*a*]. As a method, however, for routine application this offers certain difficulties. For different nerves the required concentration varies somewhat and there is no way of determining in advance the concentration necessary for any particular

TABLE IV. Change in threshold (volts) with catelectrotonus, as percentage of the threshold without electrotonus, the effect of soaking in potassium-rich Ringer's solution on this percentage and the reversibility of this effect

Exp. no.	Polarizing current μA.	Before potassium	After potassium	After potassium followed by normal Ringer's solution
1	2	−16	2	−28
	5	−26	−2·6	−42
	10	−52	1·9	−45
2	1	−18	−1·4	−10
	2	−18	2·8	−22
	5	−18	11	−30
	10	−27	38	−30
3	1	−18	−1·3	−16
	2	−27	4·5	−24
	5	−44	31	−33
	10	−51	62	−37
4	1	−10	0	−14
	2	−22	0	−25
	5	−35	0·5	−39
	10	−42	4·4	−47
5	1	−15	0	−19
	2	−29	−1·2	−33
	5	−49	−1·2	−50
	10	−57	7·8	−54
6	1	−28	6	−11
	2	−29	9	−30
	5	−47	32	−36
	10	−52	125	−27

In all Tables the sign − means a fall in threshold.

nerve. The complete abolition of the effect of electrotonus on the threshold, at least for a current of not more than a few microamperes (which is of the order of magnitude for threshold of excitation), can be more easily obtained by reducing the soaking time in a potassium solution of constant concentration (ten times normal). The experiments of Table IV (with the exception of the last experiment) show the effect of soaking for 1 hour in Ringer's solution containing ten times normal potassium. The catelectrotonus effect in the experiments with fairly weak currents is almost entirely abolished. The experiments in Table IV were undertaken to test whether the effect of potassium is reversible. The fifth column gives the percentage of threshold change with electrotonus after the nerve has been soaked again in normal Ringer's solution for the same time as it had been soaked in potassium-rich solution before. The potassium effect is reversible. The last experiment was made with both soaking times 2 hours instead of 1 hour (potassium concentration ten times normal). In this experiment also the effect is found to be reversible.

In Table V are given some determinations of the factor k (calculated from the intensity-duration curve) in nerves soaked for 2 hours in Ringer's solution containing ten times normal potassium. The effect of

Table V. The effect of electrotonus on the time-factor k of excitation in nerves soaked for 2 hours in Ringer's solution containing ten times the normal amount of potassium

Exp. no.	Polarizing current μA.	k: msec.		
		Normal	Catelectrotonus	Anelectrotonus
1	5	0·45	1·1	0·32
2	5	0·48	0·59	0·33
3	5	0·80	1·07	0·54
4	1	0·50	0·55	0·46
5	5	0·35	0·44	—
6	5	0·35	0·50	—
7	5	0·30	0·48	—

catelectrotonus is the same as in normal nerve, giving an increase in factor k. As in the threshold determinations the effects of anelectrotonus in the potassium-treated nerves are less reliable than the effects of catelectrotonus. Those experiments, however, which give results that fit Hill's equation [1936*b*] show the same decrease in k as in normal nerve.

Treatment therefore of the nerve with Ringer's solution containing excess potassium reverses the effect of electrotonus on the threshold determined with long-lasting currents, while it does not affect the influence of electrotonus on the time-factor k. This supports Hill's assumption [Hill, 1936*a*] that electrotonus is a separate phenomenon from excitation.

The effect of calcium ions. Table VI gives the effect, on the threshold for long-lasting currents, of soaking the nerve in Ringer's solution containing ten times the normal concentration of calcium. The effect is the same as in normal nerves.

TABLE VI. The effect of electrotonus on the threshold for long-lasting currents in nerves which have been treated with excess calcium for relatively short times

Exp. no.	Polarizing current μA.	Soaking time hours	Threshold: volts		
			Normal	Catelectrotonus	Anelectrotonus
1	2	3	1·64	1·49	2·05
2	2	3½	3·88	2·90	5·02
3	5	5	2·95	1·26	3·28
4	2	6	3·8	2·7	5·0
5	2	7½	4·1	3·0	5·4
6	2	8	4·8	3·6	6·2
7	5	9½	2·4	2·0	3·1

Table VII gives the effect of soaking in Ringer's solution containing ten times normal calcium on the time-factor k. The results seem to indicate that the effect of electrotonus on k is either much diminished, or abolished or even reversed. Experiments therefore were carried out

TABLE VII. The effect of electrotonus on the time-factor k of excitation in nerves treated with excess calcium for relatively short times

Exp. no.	Polarizing current μA.	Soaking time hours	k: msec.		
			Normal	Catelectrotonus	Anelectrotonus
1	2	3	0·48	0·33	0·29
2	2	3½	0·18	0·25	0·18
3	5	5	0·53	0·51	—
4	2	6	0·21	0·20	0·17
5	2	7½	0·21	0·21	0·20
6	2	8	0·25	0·28	0·22

with very much longer soaking times. The soaking was undertaken in a refrigerator in order to reduce the metabolism and depreciation with time. The nerves were removed from the refrigerator 2–3 hours before the actual experiments, and put in a fresh solution of the same concentration at room temperature.

Table VIII gives the effect of this treatment on the threshold for long-lasting currents. The influence of electrotonus on the threshold for long-lasting currents is the same as in normal nerve. Table IX, however, which gives the effect of prolonged calcium treatment on k, tells a different story. After this treatment catelectrotonus gives a decrease in k instead of an increase as in normal nerve. The influence of anelectrotonus is again less clear-cut than that of catelectrotonus. Those experiments which gave reliable results showed either an increase or a decrease

TABLE VIII. The effect of electrotonus on the threshold for long-lasting currents, in nerves treated with excess calcium for relatively long times

Exp. no.	Polarizing current μA.	Soaking time hours	Threshold: volts		
			Normal	Catelectrotonus	Anelectrotonus
1	5	12½	1·58	1·43	—
2	5	14	2·42	1·73	4·0
3	5	15	2·38	1·60	3·98
4	5	15	2·52	1·88	3·35
5	5	15	3·6	2·68	4·72
6	5	16	2·7	2·02	3·92
7	5	17	2·24	1·79	3·16
8	5	17	2·35	1·76	3·6
9	5	19	2·48	2·13	—

TABLE IX. The effect of electrotonus on the time-factor k of excitation in nerves treated with excess calcium for relatively long times

Exp. no.	Polarizing current μA.	Soaking time hours	k: msec.		
			Normal	Catelectrotonus	Anelectrotonus
1	5	12½	0·70	0·41	—
2	5	14	0·60	0·34	0·58
3	5	15	0·48	0·34	—
4	5	15	0·47	0·32	0·47
5	5	15	0·48	0·43	0·62
6	5	16	0·43	0·32	0·48
7	5	17	0·51	0·35	0·45
8	5	17	0·45	0·32	0·52
9	5	19	0·63	0·42	0·80

in k instead of the regular decrease as in normal nerves. We conclude, therefore, that prolonged calcium treatment does not alter the effect of electrotonus on the threshold for long-lasting currents, but reverses the effect of catelectrotonus on k.

The effect of cæsium ions. The results of the experiments with nerves soaked for a certain time in Ringer's solution containing cæsium chloride are given in a somewhat different form, to show the effect more clearly, in Tables X and XI. In Table X is given the effect of anelectrotonus, in Table XI that of catelectrotonus. The molar concentration of cæsium chloride was ten times that of potassium chloride in normal Ringer's solution.

TABLE X. The effect of anelectrotonus on the threshold and time-constant k of nerves treated with cæsium ions. Polarizing current 2 μA.

Exp. no.	Threshold for long-lasting currents: volts		k: msec.	
	Normal	Anelectrotonus	Normal	Anelectrotonus
1	1·0	1·9	0·53	0·37
2	1·1	2·3	0·76	0·26
3	1·0	2·4	0·39	0·29
4	0·8	2·1	0·23	0·20

TABLE XI. The effect of catelectrotonus on the threshold and time-constant k of nerves treated with cæsium ions

Exp. no.	Polarizing current μA.	Threshold for long-lasting current: volts		k: msec.	
		Normal	Electrotonus	Normal	Electrotonus
1	2	1·14	1·16	0·53	0·54
2	2	0·56	0·56	0·85	0·85
3	5	0·45	0·44	0·85	0·85
4	5	0·62	0·61	0·83	0·83
5	5	0·63	0·63	0·33	0·33
6	5	0·58	0·57	0·72	0·72
7	5	0·58	0·57	0·72	0·72
8	5	0·79	0·78	0·59	0·59

In Table X the effect of anelectrotonus is very much the same as in normal nerves. The effect, however, of catelectrotonus is quite different (Table XI). Cæsium abolishes altogether the effect of catelectrotonus both on the threshold for long-lasting currents and on the time-factor k. In fact the intensity-duration curves with and without catelectrotonus are identical within the limits of error of the determinations.

The effects of lithium and rubidium ions. Tables XII and XIII give the effect of electrotonus on nerves soaked for 2 hours in Ringer's solution

TABLE XII. The effect of electrotonus on the threshold for long-lasting currents in nerves treated with lithium or rubidium ions

Exp. no.	Polarizing current μA.	Threshold: volts		
		Normal	Catelectrotonus	Anelectrotonus
Lithium:				
1	2	1·2	0·7	2·3
2	2	1·8	0·9	3·1
3	5	1·3	0·6	4·5
4	5	1·7	0·7	4·5
Rubidium:				
5	2	1·1	1·0	1·8
6	2	1·2	1·2	1·8

TABLE XIII. The effect of electrotonus on the time-factor k of excitation in nerves treated with lithium or rubidium ions

Exp. no.	Polarizing current μA.	k: msec.		
		Normal	Catelectrotonus	Anelectrotonus
Lithium:				
1	2	0·60	0·80	0·34
2	2	0·45	1·00	0·30
3	5	0·48	1·00	0·19
4	5	0·41	0·62	0·20
Rubidium:				
5	2	1·4	2·0	—
6	2	0·74	0·95	0·62

containing excess of either lithium or rubidium ions. The molar concentration chosen was ten times that of potassium in normal Ringer's solution. The effect of electrotonus on the lithium-soaked nerve is the same as on normal nerve. The quality of the nerves treated with rubidium soon becomes very poor and therefore only a couple of experiments were performed.

Part III. *The effect of inter-electrode distance on electrotonus*

The effect of inter-electrode distance in nerve excitation is well known. With decreasing distance the threshold for long-lasting currents increases [Rushton, 1927, 1932, 1934], and the time-factor k decreases [Cardot & Laugier, 1914*b*; Bouckaert & Katz, 1935; Hill, 1936*b*]. The influence therefore of inter-electrode distance on the effect of electrotonus was investigated. The electrical circuit was as described above. A special form of wooden electrode was used which allowed very short inter-electrode distances to be used. Table XIV shows the change in threshold

TABLE XIV. The influence of inter-electrode distance on the change (±) of threshold produced by electrotonus (an. and cat.). Change of threshold expressed as percentage of threshold without electrotonus. All data are averages from six experiments

Polarizing current μA.	Short distance			Medium distance			Long distance		
	Dist. mm.	Catelec.	Anelec.	Dist. mm.	Catelec.	Anelec.	Dist. mm.	Catelec.	Anelec.
½	1½	− 3	3	14	−13	14	25	−14	14
1		− 4	4		−23	26		−19	29
2		− 5	5		−24	47		−27	56
5		−10	10		−46	130		−44	150
10		−21	27		−58	160		−58	210
20		−36	49		−63	170		−56	220
50		−51	85		−29	300		− 2	300
100		−65	85		−31	490		—	—

with electrotonus as a percentage of the threshold without electrotonus. It is seen that with short inter-electrode distance the effect of electrotonus is considerably reduced.

Rushton [1934] has shown that the increase in threshold with decreased inter-electrode distance can be explained by the difference in current distribution in the nerve with different inter-electrode distances. It was therefore investigated whether this same difference in current distribution may be responsible for the effect of inter-electrode distance on electrotonus. The experiments were performed as follows. The threshold was determined for short and long inter-electrode distance and the polarizing current was made proportional to this threshold. A comparison of columns B and C of Table XV shows that for *equivalent*

TABLE XV. The effect of inter-electrode distance on catelectrotonus if the polarizing current is made proportional to the rheobase for each inter-electrode distance

Exp. no.	Electrode distance mm. Short	Long	Polarizing current for short inter-electrode distance: μA.	A	B	C	C′
1	1	12	21	4·8	−42	−41	−90
			100		−90	−90	+23
2	1	12	21	4·8	−45	−41	−90
			100		−93	−90	−63
3	½	12	13·1	7·6	−30	−28	−83
			100		−83	−83	+42
4	1	12	16·2	6·2	−41	−39	−75
			100		−90	−75	+33
5	½	20	14·7	6·8	−15	−15	−50
			100		−50	−50	−29
6	½	20	16	6·3	−20	−18	−54
			100		−55	−54	−78

Col. A gives the ratio of the threshold for short and long inter-electrode distance.
Cols. B and C give the percentage change in threshold effected by electrotonus (− indicating decrease, + indicating increase), B for short and C for long inter-electrode distance, for polarizing currents in the same ratio as the respective thresholds.
Col. C′ gives (for comparison) the percentage reduction in threshold for long inter-electrode distance when the polarizing current is the same as for short distance (C).

polarizing currents (i.e. proportional to the respective thresholds) the percentage effect of catelectrotonus on the threshold is exactly the same for long as for short inter-electrode distance. For *equal* polarizing currents, however (compare columns B and C′), the percentage effect of catelectrotonus may be quite different in the two cases. If therefore the strength of the current producing electrotonus is made proportional to the threshold, the influence of inter-electrode distance on the effect of electrotonus on the threshold disappears completely.

It seems probable then that the effect of inter-electrode distance on electrotonus is due to the same current distribution in nerve as is responsible for the effect of inter-electrode distance on the threshold. This conclusion is a natural one if excitation is produced by current crossing a membrane and if electrotonus affects the state of that membrane.

In the experiments given in Table XIV electrotonus and excitation were applied to the nerve through the same set of electrodes. It was found that inter-electrode distance has a marked influence on the effect of electrotonus. The question remained to be investigated whether or not a constant electrotonus has any influence on the effect of inter-electrode distance on excitability. The electric circuit for the electrotonus was separated from the excitation circuit. One electrode of the electrotonus

circuit was common with the cathode of the stimulating circuit. The inter-electrode distance of the stimulating electrodes was variable, the cathode remaining in a constant position. The electrodes for the electrotonus were fixed, one of them being the same as the cathode of the stimulating current. The inter-electrode distance of the electrotonus electrodes was larger than the long inter-electrode distance of the stimulating electrodes. The results (averages of five experiments) are given in Table XVI. The difference in threshold with short and long inter-electrode

TABLE XVI. The influence of electrotonus on the effect of inter-electrode distance on the threshold for long-lasting currents. The difference in threshold with inter-electrode distance is expressed as percentage of the threshold for long inter-electrode distance. The values given are averaged from five separate experiments

Electrode distances: mm.			Polarizing current μA.	Percentage change		
Short	Long	Electrotonus		Normal	Catelectrotonus	Anelectrotonus
1	13	19	1	260	250	250
			10	260	240	210
			20	260	230	190
			40	260	170	170
			70	230	100	180
			100	190	50	170

distance expressed in percentage of the threshold for long inter-electrode distance is given. It is seen that a strong constant electrotonus gives a decrease of the effect of inter-electrode distance on excitability. The influence is stronger in catelectrotonus than in anelectrotonus.

The results described in Table XIV therefore are due to two influences, first the effect of inter-electrode distance on electrotonus and second to the influence of a constant electrotonus on the effect of inter-electrode distance on excitability.

SUMMARY

Catelectrotonus increases the time-factor k of nerve excitation, anelectrotonus reduces it.

Soaking in excess of potassium reverses the influence of catelectrotonus on the threshold for long-lasting currents. This effect is reversible. Potassium treatment leaves the effect of electrotonus on the time-factor k unaltered.

Prolonged treatment (about 15 hours) with excess of calcium leaves the effect of electrotonus on the threshold for long-lasting currents unaltered. The effect of catelectrotonus on the time-factor is reversed, while the effect of anelectrotonus is rather variable.

Cæsium ions abolish the effect of catelectrotonus both on the threshold for long-lasting currents and on the time-factor k, leaving the effect of anelectrotonus unaltered.

Lithium ions do not change the effect of electrotonus either on the threshold for long-lasting currents or on the time-factor k. Rubidium ions damage the nerve so much that reliable results could not be obtained.

Decrease of inter-electrode distance diminishes the effect of electrotonus on the threshold for long-lasting currents. If, however, the polarizing current is made proportional to the rheobase for each inter-electrode distance, this effect disappears. A strong electrotonus with constant inter-electrode distance diminishes the effect of inter-electrode distance on the threshold for long-lasting currents.

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PHARMACOLOGICAL EXPERIMENTS ON MAMMALIAN VOLUNTARY MUSCLE, IN RELATION TO THE THEORY OF CHEMICAL TRANSMISSION

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It was recently shown by Brown *et al.* [1936] that a very small dose of acetylcholine, injected directly into the empty blood vessels of a normal mammalian muscle, causes a quick contraction of the muscle, resembling a rather slow, single twitch. This contraction has now been shown by electrical records [Brown, 1936] to be a short, asynchronous tetanus, and not a contracture. It was also shown that, under suitable conditions, a small dose of eserine greatly potentiates the response of a mammalian muscle to a single maximal volley of impulses in its motor nerve, and that this potentiated "twitch" is also due to a repetitive response of the muscle, causing a brief, waning tetanus. It was suggested that these observations support the conception of a chemical transmission of excitation from motor nerve to voluntary muscle, by the release of acetylcholine at the nerve endings. The object of our experiments has been to extend these observations, and to test further their bearing on this theory.

The longer known effect of acetylcholine on denervated mammalian muscle, producing a response which is largely, at least, a contracture, was shown by Dale & Gasser [1926] to belong to that group of its actions which resemble those of nicotine [Dale, 1914]. It was of interest to know whether acetylcholine produced the rapid response of normal mammalian muscle in virtue of the same nicotine-like activity. We have therefore tested for this effect a number of choline esters and other substances having a "nicotine" action, and one choline ester of which the action is almost entirely of the "muscarine" type. Brown *et al.* attributed the potentiating action of eserine to inhibition of cholinesterase at the motor nerve endings. We have accordingly examined a series of synthetic

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substances, for which we are indebted to Dr Stedman, known to possess the inhibitory action on cholinesterase in different degrees, some being stronger and others weaker than eserine itself. We have also further examined the conditions which are required for the production of this potentiating action by eserine and its synthetic analogues, and have compared them with those under which veratrine produces a superficially similar effect.

Methods

The methods employed throughout this investigation have been essentially the same as those used by Brown *et al.* Cats and rabbits were used, under various anæsthetics, or spinal or decerebrate. The gastrocnemius muscle or the tibialis anticus was used for recording, the muscle being arranged to pull vertically on an isometric lever, with the femur or tibia and fibula immobilized by drills attached to the heavy iron bedplate of the apparatus. Muscles with natural circulation were used throughout, and injections were made, by the method earlier described in detail [Brown *et al.* 1936], into the gastrocnemius by means of a cannula in the tibial artery, the popliteal artery being occluded at the moment of injection. We have found this method to be particularly useful, not only for the rapid injections of choline esters in small amounts, but also for the direct introduction into muscle of substances which, if injected into the general circulation in sufficient amount to affect the muscle, would be lethal or very toxic to the animal under experiment. This technique, moreover, allows of more precise determination of threshold doses and, by the more rapid removal of the substance from the muscle, permits a number of observations to be made on the same preparation.

In most experiments the motor nerve to the muscle was stimulated continuously by maximal break induction shocks at a frequency of 1 in 10 sec., timed by a Lewis rotating contact breaker. When injections of a stimulating substance were made, one stimulus was omitted from the series, and the injection was made at the time when it would have occurred.

The substances used were injected in a volume of 0·5 c.c. of saline acidified to *p*H 4 with HCl—this solution by itself being without action on the muscle in this volume.

In all experiments in which injections of large doses of choline esters or of inhibitors of cholinesterase were made, the circulatory effects were avoided by previous intravenous administration of 1 mg. of atropine to the animal.

Results

(1) *Unstable choline esters*

The property of the acetyl-ester of choline in producing quick contractions of normal skeletal muscle is shared by the other unstable esters. In Fig. 1 is shown the response of the gastrocnemius of the cat to close arterial injections of butyryl-, valeryl- and propionylcholine, compared with that of 5γ of acetylcholine. It is seen that these substances produce a transient muscle twitch a little weaker in tension than that produced by acetylcholine. Like the latter, the injection is without effect upon the subsequent contractions of the muscle in response to maximal nerve stimulation. This transience of action is distinctive of the unstable esters which, in the absence of eserine, are rapidly hydrolysed by the muscle esterase.

Fig. 1.

Fig. 2.

Fig. 1. Cat, chloralose 2·4 kg. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals. A, 5γ acetylcholine by arterial injection, during intermission of one shock to nerve; B, 5γ butyrylcholine; C, 5γ valerylcholine; D, 3·5γ propionylcholine.

Fig. 2. Same experiment as Fig. 1. A, arterial injection of 50γ carbaminoylcholine; B, 7½ min. after injection.

(2) *Stable choline esters*

Brown *et al.* have shown that any process which prevents the rapid removal of acetylcholine from the muscle, when its immediate action is over, gives rise to a more or less prolonged depression of the response of the muscle to nerve stimulation. This persistence of acetylcholine was brought about by either eserine, or by the slowing of the circulatory exchange of the muscle when œdema had developed after prolonged perfusion. Should the effect, in fact, be due to the persistence of the choline ester after its exciting action is over, then the injection of a stable ester might be expected to produce such an effect in the muscle with natural circulation, and in the absence of eserine. Such conditions are fulfilled by carbaminoylcholine. In Fig. 2 is shown the effect of the

injection of 50γ of carbaminoylcholine. Its stimulant action is very weak, but it is followed by a long-lasting progressive depression of the response of the muscle. This "curarization" in the particular experiment illustrated was complete in 12 minutes and was apparently irreversible.

Acetyl-β-methylcholine is completely without action upon normal muscle. In doses from 50γ to 1 mg. it caused neither contraction of the muscle at the moment of injection, nor any subsequent depression. This is in accordance with Simonart's finding [1932] of the absence of a nicotine effect of this substance on the ganglion cell.

(3) *Other substances*

Choline itself shows, but to a very weak degree, the activity on muscle of its esters. An injection of 1 mg. produced a just visible contraction, and 5 mg. produced a rather slow contraction approximately

Fig. 3. Cat, chloralose. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals. A, arterial injection of 10γ acetylcholine during intermission of one shock to nerve; B, 5 mg. choline; C, 10γ acetylcholine; D, 0·5 mg. nicotine tartrate.

equal in tension to that following injection of 5γ acetylcholine (Fig. 3 A and B). Its activity is therefore about 1/0000th of that of acetylcholine, a relation very close to that obtained with other tissues responsive to choline and acetylcholine. The contraction produced by choline is followed by some depression of the muscular response to nerve stimulation. This depression reaches its maximum in 40–50 sec. and is followed by slow recovery, complete in some 5 min.

A substance closely allied to choline chemically, and possessing the property of exciting the contracture of denervated muscle, is tetramethylammonium iodide [Dale & Gaddum, 1930]. This substance acts on normal muscle in a way almost identical with that of choline. Arterial injection of 0·5 mg. produces a twitch similar to the response to about

7γ acetylcholine, followed by a gradually developing depression and subsequent recovery of the motor nerve twitch, exactly like that following injection of choline.

Nicotine itself has a surprisingly weak effect. In amounts up to 100γ it is without any apparent effect. A quick injection of a dose of 0·5 mg., however, is followed by a rapid contraction equal in tension to that produced by 5γ acetylcholine. The contraction is followed by a steady depression (Fig. 3). This depression is remarkable, in that it is removed completely by the stimulation of the nerve for a short time with a tetanizing current, and equally effectively by the arterial injection of sufficient acetylcholine to produce a "twitch" of tension approximately equal to that produced by the tetanic stimulation.

(4) *Action of eserine*

A number of experiments were made to elucidate certain points in connexion with the potentiation, by eserine, of the response of muscle to single maximal motor nerve volleys.

Source of acetylcholine. Although there is every reason to believe that eserine owes its effects to protection of the acetylcholine liberated at the motor nerve endings, it appeared possible that part, at least, of the action might be accounted for, if cholinergic sympathetic fibres were present in the nerve, and if acetylcholine liberated by the stimulation of these could, when protected by eserine, produce an effect upon the muscle. This possibility was tested by treating with eserine a muscle deprived of its sympathetic innervation. The lumbar sympathetic chain of one side was removed aseptically from a cat under ether, together with the first three sacral ganglia on both sides. After 10 days had elapsed the gastrocnemius was prepared and eserine was given. The effect of small or large doses was identical with that occurring in the completely innervated muscle (Fig. 4).

Frequency of stimulation. A few preliminary experiments have been made on the relation between frequency of stimulation and the reaction of the muscle to eserine. As shown by Briscoe [1936], a muscle poisoned with eserine is unable to maintain a contraction at frequencies of stimulation between 75 and 150 per sec. It behaves in many ways like a muscle under the influence of a small dose of curare. This effect appears to be due to the accumulation, in such a muscle, of the acetylcholine liberated by the nerve impulses, and to be analogous to that following injection of acetylcholine after eserine. In fact, in the presence of eserine or of any other inhibitor of choline esterase, such as nicotine, a short period of tetanic stimulation, interpolated in a series of maximal single

stimuli at intervals of 10 sec., reproduces with great fidelity the effect of an injection of acetylcholine similarly interpolated (Fig. 5). It is obvious from the long duration of the depression following a short

Fig. 4.

Fig. 5.

Fig. 4. Spinal cat, 9 days after lumbosacral sympathectomy. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals. A, arterial injection of 5γ eserine; B, 10 min. later; C, arterial injection of 20γ eserine.

Fig. 5. Spinal cat. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals, after arterial injection of 100γ miotine. A, arterial injection of 10γ acetylcholine; B, short tetanic stimulation of nerve.

Fig. 6. Cat, chloralose 2·9 kg. Contractions of tibialis anticus in response to maximal shocks to nerve. Various frequencies of stimulation. A, 6 per min. changed to 180 per min.; B, 6 per min. At arrow, intravenous eserine 0·5 mg. per kg.; C, frequency changed from 6 per min. to 180 per min.; D, frequency changed from 6 per min. to 96, 48 and 24 per min.

tetanus, that, if a frequency of stimulation is chosen which is slightly too rapid, the effect of eserine may appear, not as a potentiation of the muscle twitch, but as a depression. The effects of frequency of stimulation are shown in Fig. 6. Stimulation was started at a frequency of 6 per min., a

large dose of eserine was given and the usual potentiation appeared. Increase of the frequency up to 180 per min. now produced a fall of the tension below the original level, and from this depression the muscle did not completely recover. At a frequency of 48 per min. the depression is less pronounced; but even at the relatively slow rate of 24 per min. the tension of each twitch is still less than when stimulation is at 6 per min.

Dosage. We have made some observations on the effects of varying doses of eserine, using the method of local arterial injection for the administration of the drug only to the muscle under experiment. The threshold dose of eserine for the potentiation appears to be about 5γ in the cat's gastrocnemius. With this dose a small, obvious potentiation was regularly produced (Fig. 4). With increasing doses the potentiation increases *pari passu* until an amount of 25γ is reached. With doses in excess of this, e.g. 100γ, the potentiation begins as usual, but the rise is cut short after some 30 sec. and the muscle response falls to a level little above its previous value. In such a muscle the paralysing effect of a short tetanus, or of an injection of acetylcholine, is particularly obvious. The failure of the potentiation after large doses of eserine is probably to be attributed to the accumulation of acetylcholine, but it is difficult completely to exclude a paralysing action of eserine itself; against such an action is the fact that large doses of eserine have no depressant action *per se*, provided that the stimulation is sufficiently infrequent and that no acetylcholine is injected.

Effect of anæsthetics. Brown *et al.* showed that the eserine potentiation does not appear in the cat anæsthetized with ether. This we have been able to confirm and we have found, moreover, that even the injection directly into the arteries of a muscle of doses up to 20γ is without effect under such conditions (Fig. 8A). Similarly, we have failed to observe any potentiation by intravenous eserine in a rabbit anæsthetized with avertin, 0·3 g. per kg. Brown *et al.* suggested that the failure of Rosenblueth *et al.* [1936] to observe the potentiating action of eserine might be due to their use of dial as an anæsthetic. In our experience the effect of eserine can be obtained regularly in animals anæsthetized with dial (0·8 c.c. per kg. intraperitoneally), but the onset is slow, the maximum being attained only 10 min. after the injection, and the total effect is weaker than in the spinal animal. Nevertheless, the potentiation is typical and electrical records show the characteristic repetitive nature of the response of the muscle to single nerve volleys. Amytal similarly depresses the potentiation, and in one experiment we observed that

the administration of amytal (50 mg. per kg.), during a potentiation in a spinal cat, reduced the tension developed by each contraction.

The effects under urethane anæsthesia are peculiar. In rabbits and cats under urethane (1·5 g. per kg. subcutaneously), intravenous injection of eserine neither potentiates the mechanical response nor produces the repetitive character of the electromyogram. If, however, the eserine is given by direct arterial injection in the usual amounts, a potentiation is produced under urethane which is only slightly less than that seen in the spinal cat.

Under chloralose anæsthesia (80 mg. per kg.) all the effects of eserine are obtainable with as great facility as in the spinal or decerebrate animal (Fig. 7). For this reason, many of our later experiments have been made with this anæsthetic. Anæsthesia with ethyl alcohol in the rabbit does not prevent the appearance of the eserine potentiation.

Effects of other alkaloids. In the course of the experiments in which nicotine and tetramethylammonium iodide were used, we observed that, after these substances had been injected, the usual effect of a subsequent dose of eserine was absent; neither its potentiating nor its depressant effects could be observed. The action of acetylcholine was also apparently unaffected by eserine under such conditions, and its injection did not produce the depressant effect which is usually seen in the muscle after eserine.

(5) *Action of other inhibitors of choline esterase*

The following substances, having a graded inhibitory action upon cholinesterase, have been tested for their action on skeletal muscle:

(1) Methylurethane of *m*-hydroxyphenyltrimethyl ammonium iodide ("meta-compound").

(2) Methylurethane α-*m*-hydroxyphenylethyldimethylamine hydrochloride (miotine).

(3) Methylurethane of *p*-hydroxyophenyltrimethyl ammonium iodide ("para-compound").

(4) Methylurethane of hordenine hydrochloride ("M.U.H.").

(5) Ergotoxine.

If the activity per unit weight of eserine as an inhibitor of cholinesterase is taken as 1, then the activity of the five above compounds will be approximately as follows: meta-compound, "the most active inhibitor available," miotine 2, para-compound 1/5, M.U.H. 1/10 [Stedman, 1936], and ergotoxine 1/1000 [Matthes, 1930].

All these substances have been found to exert an action on muscle distinguishable from that of eserine only quantitatively. The threshold

doses for a potentiating effect, determined by arterial injection into the gastrocnemius of the cat, were as follows:

Meta-compound	0·25γ	Para-compound	20γ
Miotine	2·5γ	M.U.H.	200γ
Eserine	5γ	Ergotoxine	500γ

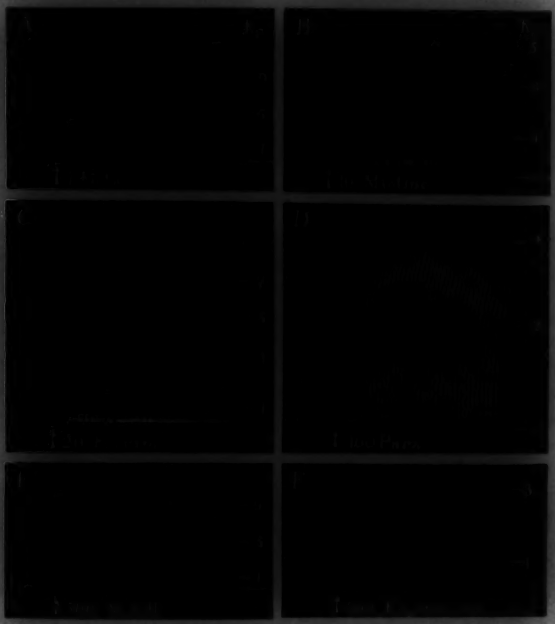

Fig. 7. Cats, chloralose. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals. Action of inhibitors of choline esterase injected arterially at the arrows. A, 1γ meta-compound; B, 10γ miotine; C, 20γ eserine; D, 100γ para-compound; E, 500γ M.U.H.; F, 500γ ergotoxine. As different strengths of myograph springs were used, reference should be made to the calibration shown on each record.

In Fig. 7 is shown a series of effects of these compounds in doses sufficient to give approximately the maximum potentiation, except in the case of ergotoxine, in which only threshold doses were given on account of the very low activity of the substance. Here again the order of the relative

activities in potentiating the muscular contraction corresponds closely to that of the relative potencies of the substances as inhibitors of cholinesterase. It is difficult to avoid the conclusion that the potentiating action is due to the prevention of the destruction of the acetylcholine liberated at the motor nerve ending. Meta-compound and miotine, on account of their high activities, have been studied in more detail. The dose of eserine which was found to be the most effective in producing potentiation, when given intravenously, was 0·3 mg. per kg. [Brown *et al.*]. Meta-compound in doses of 0·03 mg. per kg. intravenously to the cat, and miotine in doses of 0·15 mg. per kg. in both the cat and the rabbit, produce a potentiation equivalent to that of the above-mentioned dose of eserine. Excessive doses of miotine (100γ intra-arterially) or of meta-compound (5–10γ) produce very clearly the state of excessive sensitivity to the depressant action of rapid nerve stimulation, and of injection of acetylcholine (Fig. 5). With such doses of these compounds, as with large doses of eserine, the potentiation, even with such a low frequency of stimulation as 6 per min., may be irregular.

Brown *et al.* have shown that eserine is without effect upon the denervated or curarized muscle stimulated directly. This applies also to the meta-compound (Fig. 9), doses up to 25γ by arterial injection being completely ineffective. Meta-compound, therefore, like the other inhibitors of esterase, and unlike veratrine (see later), acts only when the transmitting mechanism is intact. Meta-compound appears to differ from eserine in that the effect of successive weak doses is additive when the effect of the first dose has apparently passed off. Dr Stedman informs us that the para- and meta-compounds are themselves destroyed gradually by the esterase, and have accordingly a more transient action than the other inhibitors. This applies also to their potentiating action on the response of muscle to nerve stimulation (Fig. 7).

It is to be noted that there is some discrepancy between the activity of M.U.H. as an inhibitor of esterase and the threshold dose required for potentiation. Dr Stedman informs us that the action of M.U.H. on the esterase takes a long time to attain its maximum, and this peculiarity probably explains the necessity for the use of proportionately larger doses of this compound, in relation to its final anti-esterase action, than of the other compounds with a relatively rapid action.

(6) *The action of veratrine*

Veratrine, administered to a muscle by arterial injection in doses of 15–25γ, produces a potentiation of the response to maximal single motor

nerve volleys which bears a suggestive resemblance to the action of the esterase inhibitors (Fig. 8B). On the other hand, veratrine, in concentrations of $1:10^{-4}$, has no detectable action on cholinesterase. Unlike

Fig. 8. Cat, ether. Contractions of gastrocnemius in response to maximal shocks to nerve at 10 sec. intervals. A, arterial injection of 20γ eserine; B, arterial injection of 25γ veratrine.

Fig. 9. Cat, chloralose. Contractions of gastrocnemius, denervated 9 days previously, in response to direct maximal shocks. Arterial injection of A, 5γ "meta"-compound; B, 25γ veratrine.

eserine, again, it does not sensitize the isolated rectus abdominis of the frog to acetylcholine. The resemblance in the potentiating effects, however, is superficial: the action of veratrine, when carefully analysed, is

found to be entirely different from that of the esterase inhibitors. It acts equally well on the muscle of an animal anæsthetized with ether, when eserine, even in large doses, is ineffective (Fig. 8). Denervation of the muscle (9 days after nerve section) and complete curarization do not alter the action of veratrine on it, the potentiation of its responses to direct stimulation being obtained with as great facility as that of the normal muscle to nerve stimulation (Fig. 9). The optical isometric myogram has a form which is distinctive, and quite unlike that after eserine (Fig. 10).

Fig. 10. Optical isometric myograms and action potentials of cat's gastrocnemius. A, normal response to single maximal shock to nerve. Electrical lead, belly to tendon; B, 1 min. after arterial injection of 15γ veratrine. Higher amplification of action potential; C, 1 hour later, 5 min. after a second arterial injection of 15γ veratrine. Concentric needle electrode. Time 10 msec.

The electromyogram shows that the veratrine potentiation is due to repetitive contraction of the muscle fibres, but the character of the discharge is unlike that under eserine. The action potential recorded in Fig. 10 B does not show the series of synchronous waves which is so characteristic of the eserine effect. With belly to tendon leads only a small irregular shift of the base-line after the main deflection is seen. Stricter localization of the electrical lead, as with concentric needle electrodes,

reveals that this shift is due to the asynchronous firing of small fibre groups (Fig. 10 C).

The contrast between these records and those of the muscle after eserine is obvious if Fig. 10 of this paper is compared with Figs. 12 and 13 of the paper by Brown *et al.* [1936], in which identical methods of recording were used.

Discussion

From these experiments it is clear that the property of exciting normal mammalian voluntary muscle is common, not only to the unstable esters of choline, but also to other substances possessing a nicotine-like action, viz. choline, nicotine and tetramethylammonium iodide. A similarity between the pharmacological affinities of denervated striated muscle and those of the cells of the sympathetic ganglia had long been recognized. This similarity now appears to apply also to normally innervated muscle, the only discrepancy being in the relative sensitivities of the two tissues to certain exciting substances. For instance, Feldberg & Vartiainen [1934] found that nicotine was approximately as effective as acetylcholine as a stimulant of ganglion cells. In the muscle, nicotine, in order to produce a contraction of equal tension to that produced by an injection of acetylcholine, must be injected in a dose about 200 times as great.

All the substances which possess the property of stimulating muscle to contraction have also a secondary, paralysing action, if they are allowed to persist in contact with the muscle fibres. This paralysing effect is absent in the case of the unstable choline esters in ordinary circumstances, on account of their rapid hydrolysis. Prevention of their destruction, by eserine or œdema, allows this action to become evident [Brown *et al.*]. The stable carbaminoyl-ester, choline itself, nicotine and tetramethylammonium iodide, all show to a greater or less degree the depression following excitation. The peculiar condition observed after nicotine, in which either a short tetanic stimulation or an injection of acetylcholine removes the depression, we are unable to explain; but the phenomenon is of interest in that it provides yet another example of the very close parallel between the effects of nerve stimulation and those of acetylcholine injection. In the course of the present experiments we have come across other examples of this close similarity, such as the depressant effects of a short tetanus and of acetylcholine on a muscle poisoned with any inhibitor of cholinesterase, and the absence of these effects after veratrine; also the absence of depression by tetanus and acetylcholine in a muscle

which has received eserine after nicotine or tetramethylammonium iodide. In only one instance is the parallelism between the effects of nerve stimulation and of injected acetylcholine incomplete. When the response of the muscle to nerve stimulation is depressed as a result of the injection of choline, or carbaminoylcholine, the excitatory effect of injected acetylcholine is even much more depressed (Fig. 3). Apparently this greater susceptibility of the effect of injected acetylcholine is common to curariform paralysis by different agents, being seen with curarine itself [Brown *et al.* 1936].

We have been able in these experiments to reproduce with regularity the potentiating effects of eserine on the response of muscle to single maximal motor nerve volleys. The failure of Rosenblueth *et al.* [1936] to observe this phenomenon is not entirely due to their use of dial as an anæsthetic, as Brown *et al.* suggested. In animals under dial anæsthesia, and using a dose of 0·3 mg. to 0·5 mg. eserine per kg., we have been able to demonstrate with ease that the mechanical response is increased and that the electrical record shows the typical repetitive discharge observed in the spinal or chloralosed animal. But, in all our experiments, the frequency of stimulation was kept at 6 per minute. The use of a higher frequency of stimulation, as employed by Rosenblueth *et al.* (two pairs of make and break shocks per second), is sufficient, as shown in Fig. 6, to produce a depression, and to obscure completely the potentiating action. A further factor which probably contributed to the absence of potentiation from Rosenblueth *et al.* results is their use of rather large doses of eserine (0·3–1·0 mg. per kg.) which, as we have shown, may facilitate the appearance of depression.

In our experiments with eserine and substances of similar action we have seen that there is a very suggestive correspondence between activity in potentiating the responses of a muscle to maximal nerve volleys and strength of anti-esterase action. It was shown by Brown *et al.* [1936], in the case of eserine, that this potentiation was due to repetitive response of the muscle to a single volley; and they interpreted this as indicating that eserine produced this action by inhibiting the cholinesterase at the nerve endings, and thus allowing the acetylcholine released by an impulse to persist in a concentration sufficient to restimulate the motor end plate of the muscle fibre through several successive refractory periods. The correspondence between potentiating and anti-esterase actions over a series of compounds strongly reinforces this interpretation. It is, of course, conceivable that the correspondence might be accidental; but in that case it would be necessary to suppose that the action of these sub-

stances was on the muscle fibre, so changing its physiological condition that it would give a repetitive response to a single stimulus, as frog's muscle does under the influence of unbalanced sodium ions [Adrian & Gelfan, 1933]. We have ourselves given an example of an action of this kind in the case of veratrine, which has no anti-esterase action. But veratrine causes a similar repetitive response of the completely curarized or denervated muscle to directly applied induction shocks, whereas the substances of the eserine series have no action at all on the response of muscle to direct stimulation under these conditions. The only feature of the action of these substances which might suggest a change of the excitability of the muscle fibres themselves is the appearance of small, irregular twitchings, which they all produce. This twitching, however, is not produced in the denervated or curarized muscles, and, like the potentiation of responses to maximal nerve volleys, it is produced by the different members of the series in the same order as their anti-esterase actions. The only exception to this correspondence is provided by the aberrant case of ergotoxine, which causes twitchings out of proportion to its very weak anti-esterase and potentiating actions. For the closer analogues of eserine there seems to be good reason for attributing the spontaneous twitching, like the repetitive response to a single nerve volley, to the anti-esterase action, causing the excitation of small groups of muscle fibres by the persistence of acetylcholine liberated by random impulses passing down the divided nerve. It may be noted, further, that in the case of veratrine, which certainly increases the excitability of the muscle fibres themselves, no twitchings have been produced by the doses which we employed.

Altogether, the evidence which we have put forward in this paper is not merely compatible with the transmission of excitation from motor nerve endings to voluntary muscle fibres by liberation of acetylcholine; it is difficult to find any other conception which gives a logical significance to all the facts.

Summary

1. The excitant effects of a number of choline esters on normal mammalian muscle have been compared with those of acetylcholine.

2. The excitatory action of acetylcholine on normal mammalian muscle is a property common to other substances possessing a nicotine-like action (choline, nicotine, tetramethylammonium iodide).

3. The excitation of the muscle is followed by depression of the effect of nerve stimulation when stable substances are used (carbaminoyl

choline, choline, nicotine and tetramethylammonium iodide). This depression is similar to that produced by the unstable esters when their hydrolysis is prevented by eserine.

4. The potentiation by eserine of the response of normal mammalian muscle to single maximal motor nerve volleys has been investigated further:

(*a*) It is present in the muscle deprived of its sympathetic innervation.

(*c*) It is only demonstrable if very low frequencies of nerve stimulation are used.

(*c*) It is completely suppressed by ether and avertin anæsthesia, slowed and depressed by barbiturates, but unaffected by chloralose anæsthesia or by alcohol.

5. The potentiating actions of five eserine substitutes on the response of muscle to nerve stimulation were found to be directly proportional to their inhibiting actions on cholinesterase.

6. Veratrine causes a potentiation of the muscle response to nerve stimulation, but, unlike the inhibitors of cholinesterase, its activity is not altered by anæsthetics, denervation or curarization of the muscle.

7. These facts offer further evidence in favour of the chemical transmission of excitation from nerve to voluntary muscle in the mammal.

We wish to express our thanks to Sir Henry Dale for the help and advice which he has given us in this investigation.

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THE TOTAL OSMOTIC CONCENTRATIONS IN SERUM AND AQUEOUS HUMOUR

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The theory that the aqueous humour is a dialysate from the capillary blood has lately superseded the notion that it is a secretion by certain hypothetical ciliary glands. In an earlier study the concentrations of Na^+, K^+, and Cl^-, in the sera and aqueous humours of cats were determined; [Davson *et al.* 1936]; and mean values for the ratios: $\frac{[Na^+]_S}{[Na^+]_{Aq}} = 1{\cdot}04$, $\frac{[K^+]_S}{[K^+]_{Aq}} = 1{\cdot}08$, $\frac{[Cl^-]_S}{[Cl^-]_{Aq}} = 0{\cdot}95$, were obtained. Thus it seems that there is good reason for believing that so far as these ions are concerned there exists a Gibbs-Donnan equilibrium between the blood and the aqueous humour. If this is so there must be a difference in osmotic pressure between the plasma and the aqueous humour, due partly to the protein molecules and partly to the difference in ionic concentration existing in virtue of this equilibrium. In the present study the total osmolar concentrations in the sera and aqueous humours of cats were determined by the vapour pressure method of Hill, as modified by Baldes [1934].

It will be remembered that this method depends on the comparison of a drop of the unknown solution with drops of standard solutions, using specially constructed thermocouples. The method is accurate to 0·0005 *M*, and particularly useful when dealing with small quantities.

Experimental

Cats were anæsthetized with ether and their femoral arteries cannulated; the aqueous humour was withdrawn from an eye simultaneously with the withdrawal of blood. The blood was allowed to clot and the

serum removed. Drops of each fluid were compared successively with drops of three different standard solutions of NaCl, and the mean of the estimated total osmolar concentrations in the fluids determined. In case of loss of CO_2 during withdrawal, 5 p.c. CO_2 in O_2 was passed through the thermocouple chamber until equilibrium was obtained.

Results

In Table I are shown the results on the last six cats studied, and it may be seen that in all cases the molar concentration in the serum is greater than in the aqueous humour. The mean molar difference is $0{\cdot}0018\,M \pm 0{\cdot}0002$ (twice the standard deviation), and corresponds to a difference in osmotic pressure of 31–39 mm. Hg.

Table I

Cat no.	Molarity of aqueous humour	Molarity of serum	Difference
18	0·1758	0·1774	0·0016
19	0·1708	0·1727	0·0019
20	0·1731	0·1752	0·0021
21	0·1739	0·1756	0·0017
22	0·1715	0·1730	0·0015
23	0·1703	0·1722	0·0019
Mean	0·1726	0·1744	0·0018 ±0·0002

Discussion

If the aqueous humour is to be considered as a dialysate from the plasma, the intra-ocular pressure will be determined by the following relationship:

$$I.O.P. = C.P. - O.P.$$

I.O.P. = intra-ocular pressure.
C.P. = capillary pressure.
O.P. = the difference in osmotic pressure between aqueous humour and plasma.

Taking a value of 20 mm. Hg for the mean intra-ocular pressure of the cat, and 50 mm. Hg as an approximate estimate of the capillary pressure in the ciliary region [Duke-Elder, 1932], the difference in osmotic pressure should be of the order of 30 mm. Hg, a value which agrees quite well with the experimentally determined one. The theoretical difference in osmotic pressure of plasma with its dialysate is 26–29 mm. Hg [van Slyke, 1926], so that our value of 31–39 mm. Hg appears rather high, a fact which may be accounted for by a possible unequal

distribution of non-electrolytes [Walker, 1933; Adler, 1933]. Adler found that a mean value for the concentration of urea in the plasma of 54·9 mg./100 c.c., and for aqueous humour 46·3 mg./100 c.c. This gives a mean molar difference of 0·0014 *M*, corresponding to a difference in osmotic pressure of about 27 mm. Hg. If this figure is to be taken as true, then the total difference in osmotic pressure should amount to about 54 mm. Hg, a value much higher than any obtained by us. Whereas it seems indisputable that certain non-electrolytes are not equally distributed between plasma and aqueous humour, it seems from the measurements described here that such large differences in urea concentration as those described by Adler cannot be the general rule, unless, of course, the whole concept of a simple membrane equilibrium is abandoned.

In conclusion it may be mentioned that Gilman & Yudkin [1933], using Hill's original thermopile method, concluded from their results on dogs that the plasma and aqueous humour were isotonic, although they obtained a small difference in total concentration, that in the serum being higher than that in the aqueous humour. They attributed the difference to experimental error. There can be no doubt, however, that the difference described here is real, as we have treated the results by the methods recommended by Fisher, and have obtained a standard deviation for the estimate in molar concentrations of 0·0001 *M* whilst the mean difference was 0·0018 *M*.

We wish to record our indebtedness to Mr J. L. Parkinson of the Biophysics Department for his very useful assistance in this work; and to the Medical Research Council for personal grants to two of us (H.D. and G.H.B.), and for defraying the whole expenses of this work.

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THE STANDARDIZATION OF CORTICAL EXTRACTS BY THE USE OF DRAKES

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During the past four years the control of preparations of the hormone of the suprarenal cortex in this laboratory has been attempted by different methods. At first cats were used to determine the smallest dose of each preparation which was needed to maintain life and appetite after suprarenalectomy. Cats were maintained for periods up to 68 days, but although the results indicated that the extracts were active, they never afforded a means of comparing one extract with another, or even of ascertaining the minimum dose necessary for any one cat. If the daily dose given was too small, the condition of the cat rapidly deteriorated to a point at which the administration of large doses failed to bring recovery; dramatic changes were never seen. If the daily dose was sufficient to maintain a healthy condition, it was impossible to discover how much greater it was than the minimum dose which would have the same effect. Usually all cats had difficulty in surviving the period 17–21 days after removal of the second gland, but once this was passed, their condition improved; this improvement always gave rise to a doubt whether a fragment of cortex was slowly regenerating.

When the paper by Grollman & Firor [1933] appeared, the rat method therein proposed was adopted and has been used with various modifications until lately.

By taking account of the change in body weight only, we did not find it possible to make sufficiently great distinction between uninjected and injected animals; when, however, the figure for the change in weight was combined with a figure depending on the day of death, the difference between uninjected and injected animals became much greater. The result in an animal surviving the whole period of injections was expressed simply as the weight change; when the animal died before the injections

were finished, for each day earlier 3 was subtracted from the change in weight. Using this method tests were completed in 7 days, but the effect in injected animals was not steeply graded according to the dose, and the method was scarcely more than qualitative. Recently Schultzer [1936] has described a method of using rats which enables the relative potency of different extracts to be compared; it suffers, however, from the practical disadvantage of taking 21 days to complete. Schultzer's paper gives a short review of methods which have hitherto been used.

An advance in a new direction was made possible by the observation of Parkes & Selye [1936] that, in the duck and the fowl, adrenalectomy is rapidly fatal, the birds dying within a few hours after operation. They found that the operation was more difficult in fowls than in ducks. By injections of cortical extract they succeeded in keeping one duck alive for 7 days. Their observations suggested that by using ducks a rapid method of estimating cortical extract could be devised. Dr Selye very kindly demonstrated to me his method of removing the glands; he pointed out that in the female the ovaries are often diffuse and sometimes obscure the approach. The quantitative study now to be described has therefore been made entirely on drakes, of which over 450 have been used. There has been no difficulty in obtaining a supply of drakes at any time of the year.

Adrenalectomy in the drake

Preparation

Birds have been used from 1·3 to 2·6 kg. in weight. It is desirable, however, to use birds within the limits of 1·5–1·9 kg., and, in one experiment, as far as possible of the same variety. No period of fasting before operation has been found necessary. Each bird has received an injection of 5 mg. atropine into the breast muscle, and has been anæsthetized with a mixture of 3 parts of ether and 1 part of chloroform. This has been dropped on to a mask made from a cone of perforated metal covered with muslin. Birds differ in the ease with which they are anæsthetized; some are very resistant and never completely quiet throughout the operation. During the induction of anæsthesia the feathers are plucked from the site of operation.

Operation

The bird is secured on a wooden board by means of pieces of rubber tubing fastened to the sides. The tubing is arranged to hold the wings upwards and the legs backwards to one corner of the board. The skin

incision is made about 6 cm. long parallel to and over the last ribs, in front of the lower end of the femur. The leg is drawn away from the body wall and the muscle between two ribs is divided by the blunt points of a pair of large scissors as near the last ribs as possible. The hole is enlarged with two fingers and the two ribs are held about 4–5 cm. apart by a pair of retractors. An approach is now made towards the middle of the posterior abdominal wall, through the air sacs which are torn by long pointed forceps. The field is illuminated by a head lamp. On the right side the suprarenal gland is situated between the lungs and the kidney, which is a dark brown tissue firmly attached to the side of the

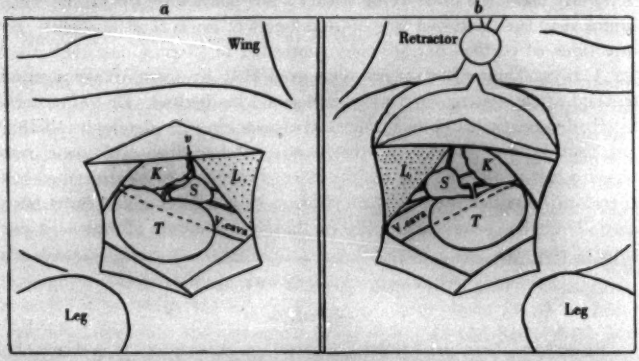

Fig. 1. Position of suprarenal gland (*S*) (*a*) on right side, (*b*) on left side. *v* = lumbar vein, *K* = kidney, *L* = lungs, *T* = testicle.

spine. The testicle, which has the shape of a kidney may be so large at some periods of the year that it covers most of the operation field; at other times it is smaller than the suprarenal gland which can be easily recognized as an orange triangular body lying upon the vena cava as shown in Fig. 1*a*. The capsule covering the gland is first removed by tearing it with the points of long straight forceps. The removal should be as complete as possible, for if incomplete, the remnants interfere with the removal of the gland itself. The vein (*v* in Fig. 1*a*) which approaches the gland from the posterior wall is gripped for 1 or 2 min. in an artery clamp; the portion anterior to the clamp is then torn, and the clamp removed; it is unnecessary to ligature the vein.

The removal of the gland is begun by rubbing it out of its bed with small pellets of cotton wool held in long forceps. If the operator works

from the posterior margin of the gland towards the vena cava, sometimes the whole gland can be loosened in this way. The gland then remains connected with the cava by the emergent vein, and this can be torn between forceps. The operation is thus complete with very little bleeding. Usually the gland cannot be entirely separated from the vein by this simple dissection, some pieces being firmly adherent to the surrounding tissue. These remaining parts are removed by suction using a vacuum pump. The suction is applied by a glass tube with an orifice about 1 mm. diameter; the edge of the orifice should be rough, so that it can be used to scrape and suck at the same time.

The amount of bleeding which occurs is variable; it is as a rule entirely venous and stops quickly, but sometimes a small artery, which runs across the hinder part of the gland, is torn; it may be necessary to ligature this. Sometimes a hole is made in the vena cava, but this can often be caught in a clamp and tied. When the gland is completely removed and all bleeding stops, the bed of the gland is moistened with cotton wool soaked in 5 p.c. ferric chloride. Careful inspection is made to ensure that no tissue is left behind, and the wound is closed. The ribs are held together by a single ligature of strong linen thread and the skin is sewn up. The bird is then turned over for the operation on the left side.

On the left side the operation is slightly more difficult because of a tail of suprarenal tissue which runs backwards underneath the capsule of the testicle for about 1 cm. Otherwise the procedure is similar to that on the right side. Arterial branches in the hinder part of the gland, and a vein between the testicle and front part of the suprarenal are the vessels which give rise to difficulty.

Operative mortality

Drakes recover from the operation quickly, and if the anæsthesia has been lightened towards the end, will drink water immediately they are put back in their cages. An inexperienced worker may take 45 min. for removal of both glands, but with practice this may be reduced to 20 min., including the preparation of the bird. Deaths occasionally occur from the anæsthetic or from loss of blood.

The survival time

Uninjected birds

Parkes & Selye [1936] give the survival time of uninjected birds as 6–20 hours. For the few hours after the operation the birds are able to stand and walk about; thereafter they sit down. The sign of ap-

proaching death is rapid respiration, and the end occurs without other warning: they die in convulsions. Four experiments to determine the mean survival time of groups of birds have been carried out at different times, and the results are given in Table I. The survival times varied

TABLE I. Survival time in hours of uninjected drakes after adrenalectomy

November 1935	January 1936	March 1936	July 1936
7·5	5·25	8·2	6·7
9·25	7·5	10·0	8·8
10·75	8·75	6·3	15·0
10·5	7·5	10·4	9·5
6·5	7·0	8·1	9·3
7·4	9·5	7·6	8·9
5·7	7·0	5·6	15·2
		10·4	12·8
			8·6
Mean 8·2	7·5	8·3	10·5

from 5·25 to 15·2 hours, the mean survival time being slightly longer in July than in November, January or March, when it was remarkably uniform at about 8 hours. When an uninjected bird has survived as long as 20–30 hours, a remnant of suprarenal tissue has been found post-mortem. Very small pieces suffice to prolong the survival for this length of time.

The time of death can be directly observed or can be recorded by means of a galvanometer and two thermocouples, one of which is held in contact with the bird's skin under its wing, while the other is placed in water in a Dewar flask. The galvanometer deflexion is continuously recorded by a "thread recorder", and when death occurs the drop in temperature under the wing is clearly indicated. There is no fall of temperature before death. My thanks are due to Prof. A. V. Hill for the loan of such an instrument with which some early observations were made; the apparatus is, however, expensive, and since it was found necessary to have an assistant present to make injections during the night, the use of the apparatus was discontinued.

Effect of sodium chloride

Two experiments were made to test the effect of sodium chloride on the survival time. In the first, three drakes were injected at 2-hour intervals with 2 c.c. of 2 p.c. NaCl given into the breast muscles. They survived 12·4, 8·6 and 10·7 hours respectively, that is to say no longer than untreated birds. In the second experiment two groups of birds were compared which were injected at intervals of 2 hours with 0·2 c.c.

of a particular extract; the birds in one group were given water to drink, while the birds in the second group were given 0·9 p.c. saline containing 5 p.c. dextrose. (The importance of carbohydrate for the adrenalectomized rat has been emphasized by Cleghorn *et al.* [1936].) In the first group there were nine drakes, and their mean survival time was 15·3 hours; in the second group there were ten drakes, their mean survival time being 14·4 hours. All birds drank much liquid, roughly 400–500 c.c.; nevertheless those drinking saline did not survive longer than those drinking water.

Effect of injections of cortical extract

Single injections of cortical extract in the breast muscle were found to have no effect in prolonging the survival time. Two birds each received one injection of 3 c.c. of an extract 4 hours after operation; they survived for 11 and 13·2 hours respectively. Three birds each received one injection of 6 c.c. of the same extract 4 hours after operation; they survived for 10·5, 11·3 and 15·2 hours respectively. All these survival times come within the range of those for uninjected birds given in Table I.

Experiments were therefore carried out in which four injections of cortical extract were given. Since the mean survival time of untreated birds was about 8 hours, it was decided to inject the doses at 1, 3, 5 and 7 hours after operation, in the expectation that an accumulation of the hormone would be obtained in the tissues and that the survival time would be prolonged in proportion to the size of the doses given. The results of an experiment in which four doses were administered to groups of drakes are recorded in Table II. The observations were made on 4 days

TABLE II. Survival time in hours of drakes injected at 1, 3, 5 and 7 hours after operation. The doses are those given at each injection. Extract A

	Dose injected (c.c.)			
	0·25	0·4	0·5	1·0
	7·75	10·05	14·2	14·1
	12·75	13·7	11·9	15·9
	12·8	9·1	13·1	12·9
	10·2	12·75	15·4	11·9
	9·8	14·2	12·2	15·6
	14·1	13·4	14·3	12·0
	11·8	—	11·7	15·7
	10·1	—	11·0	17·25
Mean	11·2	12·2	13·0	14·4

on each of which each dose was given to two or three birds. The mean survival time was prolonged in proportion to the dose, and the points lay on the curve shown in Fig. 2, in which the logarithm of the dose is used as abscissa.

The prolongation was, however, disappointingly small, and afforded no means of reasonably accurate estimation.

The standard deviations of the figures in Table II for the different means are 0·65, 0·86, 0·54 and 0·7 hours. Taking the average of these figures, which is 0·7, and applying it to the result of injecting 0·5 c.c., we see that in two out of three trials this result will lie between 12·3 and

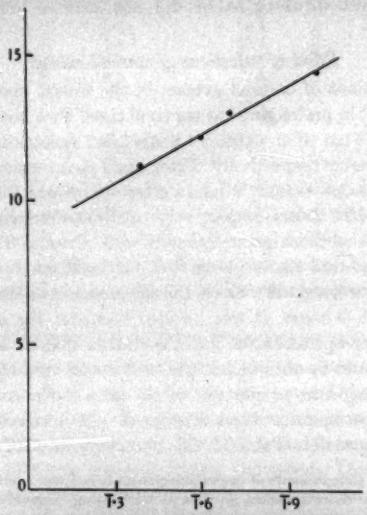

Fig. 2. Abscissæ: logarithm of dose of cortical extract injected at 1, 3, 5 and 7 hours after operation. Ordinates: mean survival time in hours.

13·7 hours, and that in twenty-one out of twenty-two trials the result will lie between 11·6 and 14·4 hours. That is to say the effect of 0·5 c.c. may be as little as that of 0·27 c.c. or as great as that of 0·93 c.c. in twenty-one out of twenty-two trials. The error is +86 or −54 p.c., the mean of which is 70 p.c. This is a low degree of accuracy.

In the next experiment injections were made at $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 hours after operation, so as to cover a longer period. Two extracts were tested, each dose being administered to a group of five to nine birds. The figures for the mean survival time are given in Table III.

TABLE III. Mean survival time in hours of groups of drakes injected at 2½, 5, 7½ and 10 hours after operation. The figure in brackets is the number of birds used in calculating the mean

	Dose at injection (c.c.)		
Extract	0·5	0·75	1·0
A	12·0 (7)	15·4 (5)	16·2 (9)
B	13·2 (7)	14·2 (8)	14·1 (7)

The figures for extract A show a slightly greater increase of survival time with increase of dose than those in Table II, but those for extract B showed almost no increase at all.

These results were disappointing and perplexing, and gave rise to the doubt that it might not be possible to use drakes for this standardization. All doses had an effect in prolonging survival time, but the difference made by a large increase in dose was negligibly small: it seemed as if the extracts prepared were unable to replace the naturally secreted hormone. The remaining possibility was that the excretion or destruction of the injected material was so rapid that there was no accumulation of the hormone in the tissues, and therefore no discrimination between the injection of 0·5 and of 1·0 c.c. If a rapid excretion or destruction actually occurred, then to prolong the survival time, repeated injections were necessary.

DETERMINATION OF A MAINTENANCE DOSE

Experiments were carried out to compare the effect of different doses of extract injected repeatedly over a long period. The time until 20 hours after operation was chosen, because it enabled observations to be completed in 2 days and required the attendance of an assistant for one night only.

In the first of these experiments injections were made 2-hourly, so that birds which survived the whole period received ten injections. The

TABLE IV. Survival time in hours of drakes injected with extract C at intervals of 2 hours after operation until death or to end of 20 hours

Dose at injection (c.c.)		
0·2	0·4	0·6
20·2	23·5	9·8
11·9	18·6	15·75
14·75	27·6	14·2
10·3	26·3	22·2
10·75	17·7	12·5
8·8	15·6	22·5
25·0	14·1	26·0
24·0	23·8	24·25
12·2	14·9	15·8
Mean 15·3	20·2	18·1

figures in Table IV give the results obtained with extract C, of which doses of 0·2, 0·4 and 0·6 c.c. were injected.

On the nine drakes injected with 0·2 c.c., five died in a period equal to 12·2 hours or less, and only three lived to receive all the injections; since one of these died at 20·2 hours it was not being maintained; hence 0·2 c.c. was a maintenance dose for two out of nine birds only. Of the birds receiving 0·4 c.c., four out of nine were maintained, and the survival time of the remainder was longer than that of birds receiving 0·2 c.c. The dose of 0·6 c.c. might have been expected to have a still greater effect; this was actually not so, for again only four birds out of nine were maintained, and the survival time of the remainder was not increased. The whole experiment was repeated with a second extract B, and gave an almost identical result. 0·2 c.c. again maintained two out of nine birds, the mean survival time of the group being 14·7 hours. 0·4 c.c. maintained five out of nine birds, the mean survival time being 20·7 hours. 0·6 c.c. maintained three out of five birds, the mean survival time being 20·6 hours. Here again it was observed that an increase of dose from 0·4 to 0·6 c.c. did not produce an increased effect.

If rapid excretion or destruction of the hormone was responsible for this failure, it seemed worth while to reduce the interval between injections to one hour; by this means a greater proportion of birds should be maintained. The results in Table V show that this was so. Using

TABLE V. Survival times of drakes injected hourly till death or 20 hours after the operation. The dose is the dose given at each injection. Extract C

	Dose at injection (c.c.)			
	0·05	0·1	0·2	0·3
	6·7	9·25	22·0	27·4
	13·5	13·75	22·8	27·75
	9·4	13·5	13·8	17·0
	8·7	15·75	17·2	21·75
	12·25	13·6	18·0	15·75
	7·75	21·25	22·75	22·3
	8·0	16·7	23·35	29·0
	9·7	18·9	25·1	25·2
	12·25	10·1	13·2	25·0
	—	—	—	24·3
Mean	9·8	14·7	19·8	23·5
Proportion maintained	0/9	1/9	5/9	8/10

extract C, the dose of 0·05 c.c. was without effect; the dose of 0·1 c.c. was sufficient to maintain one out of nine birds, and the mean survival time was increased to 14·7 hours. The dose of 0·2 c.c. maintained five out of nine birds, and the mean survival time was 19·8 hours; finally

TABLE VI. Survival time of drakes injected hourly till death or 20 hours after the operation. The dose is the dose given at each injection. Extract D

	Dose at injection (c.c.)		
	0·1	0·2	0·3
Mean survival time	11·2	19·9	24·6
Proportion maintained	0/9	5/10	5/6

0·3 c.c. maintained eight out of ten birds and increased the survival time to 23·5 hours. The experiment was repeated with extract D with results summarized in Table VI. These agreed closely with those in Table V both in respect of mean survival times and of the proportion of birds maintained. By combining the two experiments, mean figures were

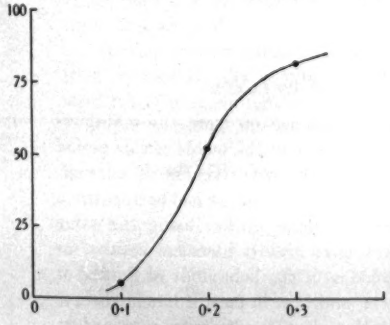

Fig. 3.

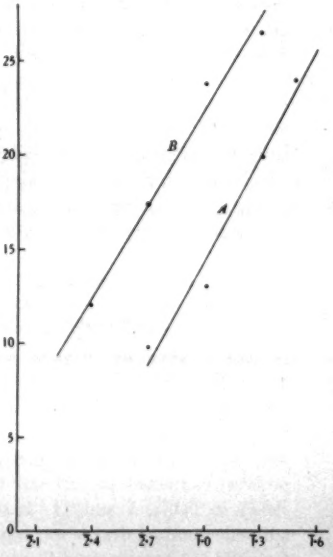

Fig. 4.

Fig. 3. Abscissæ: dose of cortical extract injected hourly for 20 hours after operation: c.c. Ordinates: percentage of birds maintained during the period of injections.

Fig. 4. Abscissæ: logarithm of the dose of cortical extract injected hourly for 20 hours after operation. Ordinates: mean survival time in hours. *A* is the line obtained by plotting the May figures in Table VIII. *B* is obtained by plotting the July figures.

obtained (see also Table VIII) which were utilized to obtain a curve relating dose to effect in two different ways.

The dose was related in the first place to the proportion of birds maintained during the period of injections without reference to the figures for mean survival time. Thus the dose of 0·1 c.c. maintained 1 out of 18, or 5·5 p.c.; 0·2 c.c. maintained 10 out of 19, or 52 p.c.; 0·3 c.c. maintained 13 out of 16, or 81 p.c. The relation of dose to percentage of birds maintained is shown graphically in Fig. 3. The dose was related in the second place to the mean survival time, and it was interesting to observe that when the points were plotted, using as abscissa the logarithm of the dose rather than the dose itself, they lay on a straight line, shown in Fig. 4 *A*. These experiments, then, in which cortical extract was injected hourly for 20 hours, finally gave a relation between dose and effect which was suitable for routine standardization. By using the formula given by Fisher [1925]

$$y-\bar{y}=b\,(x-\bar{x}),$$

where $\bar{y}$ is the mean value of y (the survival time) and $\bar{x}$ is the mean value of x (logarithm of the dose), and where

$$b=\frac{\Sigma y\,(x-\bar{x})}{\Sigma\,(x-\bar{x})^2},$$

the equation to the line *A* in Fig. 3 was found to be

$$y=18{\cdot}6x+33{\cdot}1.$$

Changes in sensitiveness of drakes

At this stage experiments had been carried on from the middle of November to the beginning of June. Throughout the whole of this period there was no suggestion of variation in the sensitiveness of adrenalectomized birds as a whole at different times; indeed, as has been pointed out, different extracts (prepared by the same worker using the same process) examined at different times gave almost identical results, as shown in Tables V and VI. It seemed as if the behaviour of groups of adrenalectomized drakes was uniform, and that in fact, if not in theory, it would be possible to define a drake unit. Results were obtained in the end of June and in July, however, which proved that a change in sensitiveness had taken place. It may be noted in passing that the mean survival time of untreated birds, found constant in November, January and March at about 8 hours, was longer in July (see Table I). The observations recorded in Table VI had already been made on extract D,

and their similarity to those in Table V had been noted. Further experiments were then made in which extract D was used as a standard of comparison for another extract, and the results then obtained with extract D are given in Table VII. On 4 June 0·2 c.c. maintained three

TABLE VII. Extract D. Dose injected hourly for 20 hours or till death

Date	Dose (c.c.)	Mean survival time (hours)	Proportion maintained
4 June	0·2	18·8	3/7
29 „	0·2	26·0	4/4
7 July	0·1	23·7	4/5
9 „	0·05	18·7	2/7
28 „	0·025	12·0	0/5
30 „	0·05	17·8	2/5

out of seven birds and gave a mean survival time of 18·8 hours. On 29 June a dose of 0·2 c.c. maintained all of four birds to which it was given; on 7 July 0·1 c.c. was sufficient to maintain four out of five birds, and on 9 July 0·05 c.c. gave a mean survival time of 18·7 hours, equal to that given by 0·2 c.c. on 4 June. The result obtained on 30 July confirmed the result of 9 July, and that of 28 July was in conformity with it, having regard to the smaller dose. The figures in Table VII are strong evidence that in the period 4 June to 9 July drakes changed in sensitiveness to such an extent that at the end of the period one-quarter of the dose initially found necessary sufficed to prolong life for a given time after adrenalectomy.

Another extract E was also tested in July in simultaneous comparison with extract D; the results indicated that the two were of the same strength. The mean figures for extracts C and D tested in May, and for D and E tested in July are given in Table VIII; the results in May are

TABLE VIII. Mean survival times (hours) of drakes injected hourly for 20 hours or till death

		Dose (c.c.)				
Extract	Date	0·025	0·05	0·1	0·2	0·3
C and D	May	—	9·8	13·0	19·9	24·0
D and E	July	12·0	17·4	23·8	36·5	—

those from which the line *A* in Fig. 3 was obtained, and the results in July are those from which the line *B* was obtained. The equation to the regression line for the July results is $y = 16{\cdot}6x + 39{\cdot}0$. The slope of the line given by the coefficient of x, which is 16·6, is almost the same as that for the regression line for the May results which was 18·6. This means that the two lines are nearly parallel.

So far as evidence is available, no change in sensitiveness took place from November to June, but only during June. At this time it was observed that the testicles became smaller, and the lessened need for the hormone was probably connected with this diminution in testicular activity at the close of the breeding season. That a change in sensitiveness occurs makes it essential to regard the method simply as a means of comparing the activity of two preparations; it does not afford a basis for defining a unit in terms of the effect of the hormone on the drake, even during the period from November to June, for although changes of sensitiveness were not noticed then, it is quite possible that they occurred.

Use of the method for routine purposes

The use of the method for routine purposes is best explained by an actual comparison of an extract T with an extract S used as a standard. On the first day on which S and T were compared, preliminary observations were made in which 0·05 c.c. of S was given to three birds because it was expected that this would produce a mean survival time of about 16–17 hours. Since T was believed to be weaker, 0·1 c.c. was given to three birds and 0·2 c.c. to three others. The results were:

	Mean survival time
0·05 c.c. S	21·9 hours (3 birds)
0·1 c.c. T	13·9 „ „
0·2 c.c. T	18·9 „ „

The general indication from this preliminary experiment was that T was less than 50 p.c. of S. The main experiment then began on the next day when five birds were injected with 0·025 c.c. S and five birds with 0·1 c.c. T. The dose of S was halved because a mean survival time of 21·9 hours approaches a maximal effect; for the same reason the dose of 0·1 c.c. T was used rather than 0·2 c.c. The result was:

	Mean survival time
0·025 c.c. S	12·0 hours (5 birds)
0·1 c.c. T	13·2 „ „

The comparison was completed on another day when 0·05 c.c. S was given to five birds and 0·1 c.c. T was given to four birds. It would have been better to have given the dose 0·2 c.c. T. The result was:

	Mean survival time
0·05 c.c. S	17·8 hours (5 birds)
0·1 c.c. T	12·4 „ (4 birds)

The results on the three days when summarized appeared as follows:

	No. of birds	Mean survival time (hours)
0·025 c.c. S	5	12·0
0·1 c.c. T	12	13·4
0·05 c.c. S	8	19·4
0·2 c.c. T	3	18·9

Evidently T was approximately 25 p.c. of S.

The precise relationship between S and T can be calculated either graphically from Fig. 4 or algebraically from the equation to the regression line. The two methods may be illustrated by taking the first pair of final observations that 0·025 c.c. S gives a mean survival time of 12 hours, and 0·1 c.c. T gives a mean survival time of 13·4 hours. The abscissæ in curve 1 corresponding to 12 hours and 13·4 hours are observed, and since these are logarithms the corresponding antilogarithms are found. The ratio of the potency of 0·025 c.c. S to 0·1 c.c. T is then equal to the ratio between these antilogarithms. That is the graphic method. The algebraic method uses the equation to the regression line which is $y = 18{\cdot}6x + 33{\cdot}1$ for curve *A*. If 12 be substituted for y then $x = -1{\cdot}134$ or $\bar{2}{\cdot}866$. This is the logarithm of 0·0734. If 13·4 be substituted for y in this equation, then $x = -1{\cdot}059 = \bar{2}{\cdot}941$. This is the logarithm of 0·0873.

Then $\frac{0{\cdot}1 \text{ c.c. T}}{0{\cdot}025 \text{ c.c. S}} = \frac{0{\cdot}0873}{0{\cdot}0734} = 1{\cdot}19$. Therefore 1 c.c. T = 0·297 c.c. S, or T has 29·7 p.c. of the potency of S.

The method as here described requires three days, if one day is taken for preliminary observations. As the example shows, however, useful information may be obtained in one day, and the succeeding days serve in the main to confirm or to correct the information obtained in the preliminary experiment. The calculation of the mean survival time from a small number of birds is often less unreliable than might be expected. In the preliminary experiment the survival times for 0·1 c.c. T were actually 20·2, 13·0 and 8·5 hours. To calculate a mean from these figures would seem to invite error; nevertheless, the mean is 13·9, which scarcely differs from the final mean of 13·4 for 12 birds.

The accuracy of the method

The accuracy of the method can be determined by calculating the average standard deviation of the mean survival time. For example in Table V the mean survival time for 0·1 c.c. is 14·7 hours.

The standard deviation of this figure is 1·44 hours determined from the formula

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

The standard deviation of the mean survival time 19·8 hours for a dose of 0·2 c.c. is 1·45 hours. The standard deviation of three other means has also been determined; the figures were 0·95, 1·6 and 1·3 hours. The average of the five figures is 1·3 hours. Taking the mean survival time of 14·7 hours in Table V for dose 0·1 c.c., we see that in two out of three repetitions of this observation the result will lie between 13·4 and 16·0 hours. But 13·4 hours is shown by Fig. 4 *A* to correspond to log $\bar{2}$·94 which is a dose of 0·087 c.c. and 16·0 hours to log $\bar{1}$·075 which is a dose of 0·119 c.c. Hence in two out of three repetitions when a dose of 0·1 c.c. is given the result will indicate not less than 0·088 c.c. or more than 0·113 c.c. to have been given. That is to say, the error will be not more than 16 p.c. Similarly in twenty-one out of twenty-two repetitions, the error will be not more than 33 p.c. These percentages are calculated for experiments in which ten birds are used to determine the mean survival time. The standard deviation of the ratio of potency between two extracts, when ten birds are used for each extract, is obtained from the formula $\sqrt{\sigma_1^2+\sigma_2^2}$, where σ_1 is the percentage standard deviation of the determination on the first extract, and σ_2 of the determination on the second extract. Here $\sigma_1=\sigma_2=16$, hence the standard deviation of the ratio is 22·6 p.c.

Discussion

It is of great importance for the manufacturer who is engaged in making gland extracts for clinical use, and also for the biochemist who is engaged in isolating active principles to have at their disposal a method which enables biological potency to be determined rapidly. The chief advantage of the method in which the drake is used for the estimation of cortical extract is that results are obtained in 24 hours, and, moreover, the accuracy of these results can be calculated precisely. The development of the method has illustrated many of the principles of biological methods in general. Drakes alter in sensitiveness between May and July, for in July they require a much smaller amount of hormone to prolong life than in May; possibly they change at other times of the year. For this reason the method must be comparative; a drake unit cannot be defined, and to standardize the different extracts made by a firm, one extract must be compared with a preceding extract.

The course of the work has further illustrated the value of determining mean effects in groups of animals to avoid the difficulties of individual variation, and also the importance of improving a method until the ratio of increase of effect to increase of dose becomes sufficiently large. The cortical hormone when administered in watery solution is rapidly destroyed or excreted by the drake. When single injections of a small series of dose are given, the distinction between the effects of small doses and of large doses is very slight. The method described is one in which hourly injections are made, and consists essentially in determining the maintenance dose over a given period for a certain proportion of birds. To calculate the potency of the extract from this proportion, however, is not the best way of using the information obtained; it is more accurate to determine the mean survival time which increases rapidly with increase of the dose given at each injection, and which has a rectilinear relation to the logarithm of the dose.

The use of cortical extracts is still very limited because of their cost. The extracts used in this research, prepared by Messrs Allen and Hanburys, Ltd., contain in 1 c.c. the activity from as much as 75 g. of suprarenal cortex. It may be that when a watery solution of the hormone is injected into a patient, it suffers the same fate as in a drake, being rapidly excreted or destroyed, and that for this reason extracts of high concentration only produce an appreciable clinical effect. If some other solvent were chosen from which the hormone was liberated slowly inside the body, a much more economical use of the extract might be possible.

Summary

1. The operation of removing both suprarenal glands in the drake is described.

2. The mean survival time of drakes after adrenalectomy is 8–10 hours, according to the time of year.

3. By giving hourly injections of cortical extract the survival time can be prolonged and if the dose is sufficient the life of drakes can be maintained.

4. A method of determining the relative potency of different samples of cortical extract is described in which 20 injections at hourly intervals are given. When two extracts are compared and each extract is given to a group of ten birds, the standard deviation of the ratio of potency is 22·6 p.c. The advantage of the method is its rapidity.

I wish to thank Prof. J. H. Burn for suggesting this problem to me, and for his advice throughout the work; also Mr H. W. Ling for his help at the operations.

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THE STATE IN THE BLOOD AND THE EXCRETION BY THE KIDNEY OF THE ANTIDIURETIC PRINCIPLE OF POSTERIOR PITUITARY EXTRACTS

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Extracts of the posterior pituitary (pituitrin), when mixed with blood or certain tissue suspensions and subcutaneously injected into rats, no longer display the antidiuretic activity of pure pituitrin [Heller & Urban, 1934, 1935]. The following evidence led to the conclusion that the inactivation of the hormone is due to adsorption: (1) The process of inactivation is very rapid. (2) The rapidity of the process of inactivation does not depend on the amount of antidiuretic hormone added. (3) The inactivation is reversible. Heating releases the biologically active hormone.

Using body fluids and tissues from several sources it was found that, for each tissue, the adsorption capacity is of specific magnitude. The number of milliunits[1] of pituitrin inactivated by a standard amount of tissue or fluid was accordingly termed its specific adsorbing capacity (s.a.c.). An inactivation of the antidiuretic principle by human serum has also been observed by Dietel [1933]. Theobald [1934] mentions a reversible inactivation of the hormone by both corpuscles and serum *in vitro*.

The s.a.c. of blood is less than that of any other tissue investigated, but it is large enough to inactivate amounts of the antidiuretic principle which are many times larger than the minimum effective dose. The means by which coagulation of the blood is prevented does not seem to make any difference to its adsorbing capacity. It seems therefore unlikely that

[1] The term milliunit is used to express the antidiuretic activity of 10^{-4} c.c. of Pituitary (Posterior Lobe) Extract B.P. The extract employed in the investigation described in this paper was Messrs Parke, Davis and Co.'s "Pituitrin".

the behaviour of circulating blood is qualitatively different from the behaviour of blood *in vitro*. On the contrary, it can be expected that addition of the antidiuretic hormone to circulating, i.e. moving, blood secures a more rapid adsorption. If we then assume that an inactivation of the antidiuretic principle takes place *in vivo* as well as *in vitro*, how are we to explain the fact that small doses of the hormone should be completely adsorbed and nevertheless have an action on the kidney? That such renal action exists has been amply demonstrated by the work of Starling & Verney [1925], Burgess *et al.* [1933] and others.

An explanation can probably be found with the help of the following findings. Heller & Urban [1935] have shown that the mammalian kidney excretes the antidiuretic hormone in an active form. The doses of posterior pituitary extract used in these preliminary experiments were so large that they transcended the S.A.C. of the blood. That is to say a considerable fraction of the injected hormone was sure to have been "free", i.e. not adsorbed by the blood colloids. If it can be shown, however, that the bound as well as the free antidiuretic hormone can be excreted, then the apparently contradictory findings, that a substance which is inactivated by the blood nevertheless has a renal action and is excreted by the kidney, can be explained by the assumption that the hormone is liberated by some unknown process in the kidney.

In this paper an attempt was made to investigate this possibility by obtaining further data about the physiological activity of small amounts of the antidiuretic factor when mixed with blood *in vitro* or when injected intravenously, and by a quantitative study of the elimination of posterior pituitary hormone by the normal kidney.

Methods

In all but one instance male rabbits were used throughout the experiments. They received a standard diet during at least a week before the beginning of the experiments. This diet consisting of oats, bran and water produced an acid urine which seemed desirable in view of the possible inactivation of pituitrin in alkaline media [Gaddum, 1930]. The animals received no food for 16 hours before the beginning of the experiments, water being allowed up to 6 hours before the experiments. For the assay of the antidiuretic potency of urine and blood samples, Burn's [1931] method of subcutaneous injection into rats was used. This method has been found to yield satisfactory results for the estimation of amounts ranging from 2 to 12 milliunits pituitrin per 100 g. rat. Further details of the method are described in a previous paper [Heller & Urban, 1935].

The antidiuretic effect is measured by the time taken to reach the maximum rate of water excretion. This will be referred to as *inhibition time*. The normal inhibition time, i.e. the delay after giving the standard amount of tap water, was found to be 86 ± 11 min. in the present series of 148 rats.

The antidiuretic activity of samples of urine was estimated in terms of doses of pure pituitrin which were found to possess equal antidiuretic action when tested on the same animals. Several groups of four rats each were standardized first with pure pituitrin. The estimations of the rabbit's urine were made two or three days later. A series of dilutions of the urine samples was prepared. 0·5 c.c. of diluted urine per 100 g. rat was injected into each of a group of four rats. Other sets of four rats each were injected with 0·5 c.c. per 100 g. rat of urine which had been diluted to different degrees. A double control is effected by this procedure. The results can first be compared with those observed after the injection of pure pituitrin a few days ago. Secondly, if sample A, for instance, is found to contain 10 milliunits per c.c. urine in the dilution 1:1, then the diuresis of rats injected with a dilution of 1:5 should be inhibited to a degree which corresponds to a concentration of 2 milliunits of pituitrin per c.c. It is thought that by this method gross errors in the assay of the antidiuretic principle were avoided. Controls were performed to make sure that mixtures of acid urine and pituitrin lose none of their activity if kept for 12 hours in the refrigerator. It should be added that an antidiuretic action of pure urine was in no case observed in our experiments.

The urine samples were obtained in the experiments with the rabbit by bimanual expression of the contents of the bladder. As a control a few animals were catheterized after this procedure, but no residual urine was obtained.

The samples of pituitary extract were kept at 0° C. The same batch of "pituitrin" was used throughout any given series of experiments. Urinary chlorides were determined by Volhard's method and urinary proteins by the gravimetric method of Folin & Denis [1914].

Results

The adsorption of the antidiuretic factor of posterior pituitary extracts by rabbit's blood in vitro

The blood was collected from the carotid arteries of rabbits anæsthetized with ethyl urethane. Coagulation was prevented either by defibrina-

tion or by addition of 0·1 g. sodium citrate to 10 c.c. of blood. 0·5 c.c. saline containing the appropriate dose of pituitrin was added to 9·5 c.c. of blood, and 1 c.c. of this mixture per 100 g. rat was then injected subcutaneously into a group of four rats.

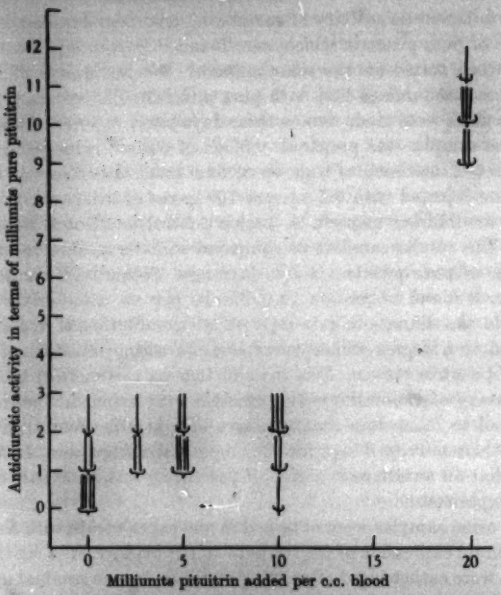

Fig. 1. Specific adsorbing capacity of rabbit's blood for the antidiuretic principle of posterior pituitary extracts. The points of the ordinate were obtained by comparing the inhibition times (see p. 83) of rats injected with blood-pituitrin mixtures with those of rats which were injected with pure pituitrin only. The antidiuretic activity of the control samples (pure blood) is shown under "0 milliunits added". For some unknown reason some of these samples had a slight antidiuretic activity, i.e. the rats had a higher inhibition time than with water alone. Each vertical line represents the equivalent in pure pituitrin of a sample of a pituitrin-blood mixture injected into a group of four rats. The length of the line indicates the probable range of error of the method.

A reference to Fig. 1 shows (1) very little or no difference between the antidiuretic activity of pure blood and mixtures of blood with pituitrin up to the concentration of 10 milliunits per c.c., and (2) an antidiuretic activity equivalent to about 10 milliunits of pure pituitrin of mixtures

of blood with 20 milliunits per c.c. Both these findings indicate approximately 10 milliunits as the specific adsorbing capacity of rabbit's blood.

Amounts of pituitrin below 10 milliunits per c.c., i.e. below the S.A.C. of rabbit's blood, therefore, should rapidly be adsorbed and inactivated after intravenous injection into a normal rabbit. Five experiments were performed. The carotid artery was exposed in ether anæsthesia and the animals were then permitted to recover. Four to six hours later 4 p.c. of the rabbit's body weight of water was given by a stomach tube. After 25–30 min. the first (control) sample of blood was obtained by puncture of the carotid artery, a local anæsthetic being employed. The pituitrin was then injected intravenously. A second and a third blood sample were taken 1–2 and 5–8 min. after the injection. The doses of pituitrin chosen were calculated to produce concentrations ranging from 5 to 10 milliunits per c.c. of circulating blood. The blood volume was assumed to be 8 p.c. of the body weight. The samples were either citrated or defibrinated. The antidiuretic potency of the blood samples was tested on two groups of four rats each.

For example, in one of these experiments subcutaneous injections of 1 c.c. per 100 g. rat of blood removed from a rabbit before the injection of the antidiuretic hormone resulted in inhibition times (see p. 83) of 142 min. in one group of four rats and 113 min. in another group of four rats. 6·5 milliunits of pituitrin per c.c. blood were then injected intravenously into the rabbit. Blood samples were taken from the carotid artery two minutes and five minutes after the animal received the injection of pituitary hormone. Injections of 1 c.c. of these blood samples per 100 g. rat were made into four groups of four rats each. The inhibition times were 97 and 127 min. after injection of the blood samples which were removed after a delay of two minutes, and 105 and 97 min. after the injection of the blood samples removed after five minutes. There is no significant difference between the antidiuretic activity of the controls and of the other blood samples. In other words the hormone was inactivated within two minutes after injection. The other experiments gave similar results in that either all or the bulk of the injected hormone was inactivated within the first two minutes after intravenous injection.

The ultrafiltrability of the antidiuretic hormone

The formation of an adsorption compound with the blood colloids implies a change in the ultrafiltrability of the antidiuretic principle. The published results of ultrafiltration and dialysis experiments as summarized in Table I are in favour of this conclusion. It will be seen that

TABLE I. Dialysis and ultrafiltration of the antidiuretic and the pressor principle of posterior pituitary extracts in solutions containing (*a*) no colloids; (*b*) serum or whole blood. A.P. = antidiuretic principle. P.P. = pressor principle (no difference in "chemical" or "physical" properties has so far been demonstrated between the antidiuretic and the pressor principle. Experiments with the latter were, therefore, included in this table)

Nature of filter used	Nature of colloid-free medium used	Results	Nature of colloid-containing medium used	Results	Author
6 p.c. collodion dissolved in alcohol-ether mixtures of varying concentrations	0·1 p.c. glacial acetic acid	A.P. and P.P. diffuses rapidly and quantitatively	—	—	Smith & McClosky, 1924
Collodion	0·25 acetic acid	P.P. dialyses rapidly	—	—	Kamm, 1928
4 p.c. collodion	Ringer solution	A.P. present in ultrafiltrate	—	—	Dietel, 1933
Collodion	?	A.P. passes freely	Citrated human plasma	Pituitrin demonstrable in ultrafiltrates of plasma containing 2 milliunits pituitrin per c.c.	Byrom & Wilson, 1934
Candles impregnated with 7 p.c. collodion in acetic acid	Acid saline	Quantitative recovery of P.P.	Plasma of *p*H 4·5	Ultrafiltrate completely destitute of pressure effect	De Wesselow & Griffith, 1934
10 p.c. collodion membrane on sintered glass Büchner filter	0·5 c.c. (5000 milliunits) pituitrin added to 5 c.c. saline	Ultrafiltrate contains less than 10 milliunits per c.c.	0·5 c.c. pituitrin and 0·5 acetic acid added to 9 c.c. blood plasma	Ultrafiltrate contains no appreciable amount of A.P.	Theobald, 1934
Ultracella filter "fein" (holding up benzopurpurin molecular weight about 800)	—	Ultrafiltrate contains considerable amounts of P.P. and A.P.	10 c.c. pituitrin were added to 50 c.c. blood of pregnant woman (200 milliunits per c.c.)	Ultrafiltrate contains less than 20 milliunits per c.c.	Theobald, 1934
10 p.c. nitrocellulose	Normal saline	Recovery in direct proportion to the volume filtered	2000 milliunits added to dog's blood (40 milliunits per c.c.)	First traces of pituitrin recoverable	Levitt, 1936

the antidiuretic hormone in a solution free of colloidal matter diffuses and filters freely through comparatively dense membranes, an indication of the low molecular weight of the "free" hormone. All but one series of observations, however, show that either no antidiuretic activity is found in ultrafiltrates of the hormone with blood or serum, or that very large doses of posterior pituitary extract must be added before the first traces of antidiuretic activity appear in the filtrates. These findings suggest strongly that the antidiuretic hormone is rendered non-ultrafiltrable by adsorption to the blood colloids.

Further ultrafiltration experiments were performed with mixtures of pituitrin and rabbit's blood. The filters used were membranes of 7 p.c. collodion in glacial acetic acid made on the surface of sintered glass Büchner filters. The concentrations of pituitary hormone used were 8 and 10 milliunits per c.c. defibrinated rabbit's blood. The antidiuretic activity of the ultrafiltrates was tested by subcutaneous injections into rats. No appreciable amounts of the antidiuretic principle were found in the ultrafiltrates of these mixtures (Table II). Pituitrin dissolved in the same volume of saline was found to pass freely through the collodion ultrafilters. An adsorption of the antidiuretic principle by the filters can, therefore, be excluded.

TABLE II. The difference in ultrafiltrability of the antidiuretic hormone in mixtures with (a) saline; (b) defibrinated rabbit's blood. Any given series of experiments was performed on the same group of rats at intervals of two days

	Inhibition time in min.
1. Rats receiving 5 p.c. of body weight of water only	68
2. Rats receiving 5 p.c. of body weight of water + 1 c.c. per 100 g. rat of the ultrafiltrate of a mixture containing 8 milliunits pituitrin per 10 c.c. of defibrinated blood	68
3. Rats receiving 5 p.c. of body weight of water + 1 c.c. per 100 g. rat of the ultrafiltrate of a mixture containing 8 milliunits pituitrin per 1 c.c. of saline	195

So far the concentration of the antidiuretic hormone in the pituitrin-blood mixtures was below the S.A.C. of rabbit's blood (see p. 85). However, free, i.e. unadsorbed, antidiuretic hormone should be present in an ultrafiltrate of blood containing an amount of pituitrin above the S.A.C. of rabbit's blood. This we were able to demonstrate in several experiments. Table III shows an example of these results.

The S.A.C. of rabbit's blood for the antidiuretic hormone can, therefore, be estimated as above 10 and below 20 milliunits pituitrin per c.c. blood. These figures are in satisfactory agreement with those obtained by the biological method of the preceding section.

TABLE III. The content of "free" pituitrin, as measured by the antidiuretic effect, of ultrafiltrates of blood samples containing (a) an amount of antidiuretic hormone assumed to be near or equal to the S.A.C. of rabbit's blood (8 milliunits per c.c.) and (b) an amount assumed to be above the S.A.C. of rabbit's blood (20 milliunits per c.c.). Any given series of experiments was performed on the same group of rats at intervals of two days

	Inhibition time in min.
1. Rats receiving 5 p.c. of body weight of water only	97
2. Rats receiving 5 p.c. of body weight of water + 1 c.c. per 100 g. rat of the ultrafiltrate of a mixture containing 8 milliunits pituitrin per c.c. of saline	225
3. Rats receiving 5 p.c. of body weight of water + 1 c.c. per 100 g. rat of the ultrafiltrate of a mixture containing 8 milliunits per c.c. of blood	113
4. Rats receiving 5 p.c. of body weight of water + 0·5 c.c. per 100 g. rat of the ultrafiltrate of a mixture containing 20 milliunits per c.c. of blood	180

The excretion of the antidiuretic hormone in the urine

Experiments on rats [Heller & Urban, 1935] have shown that a part at least of a large intravenous dose of the antidiuretic hormone is excreted by the kidney in an active state. However, neither the quantity excreted nor the time relations of the excretory process were investigated. These further details are analysed by the experiments described in the following section.

A larger animal, the rabbit, was substituted for the rats of our previous experiments. The animals were placed in metabolism cages. Either 30 or 45 min. before the injection of the antidiuretic hormone 4 p.c. of the animal's body weight of warm tap water was given by stomach tube. The antidiuretic hormone was injected into the ear vein. The urine was collected every 30 and in some experiments every 15 min.

(*a*) *Amounts of antidiuretic principle excreted after intravenous injection of doses ranging from* 1 *to* 10 *milliunits per c.c. of circulating blood.* The quantities of antidiuretic hormone excreted by the same rabbit after repeated injections of identical amounts on different days vary only little. For example: 10 milliunits per c.c. blood were injected intravenously into rabbit No. 5; 294 ± 42 milliunits or 11–15 p.c. of the amount injected were found in the urine. Three days later the animal received the same dose. 320 ± 60 milliunits or 11–18 p.c. of the amount injected were excreted. A series of experiments was then performed with doses rising from 1 to 10 milliunits per c.c. blood (Table IV). It was found that the total amounts of antidiuretic hormone excreted were almost identical. In other words the percentage of antidiuretic hormone excreted in the

TABLE IV. Excretion in the urine of the antidiuretic principle of posterior pituitary extracts by the normal rabbit after intravenous injection of doses ranging from 1 to 10 milliunits pituitrin per c.c. blood. The blood volume was assumed to be 8 p.c. of the body weight

No. of animal	Initial concentration of antidiuretic hormone per c.c. circulating blood (in milliunits pituitrin)	Total amount of antidiuretic principle excreted (in milliunits)	Percentage of antidiuretic principle excreted
2	10	212 ± 42	8–12
	2	220 ± 60	37–64
5	10	294 ± 42	11–15
	10	320 ± 60	11–18
	2	310 ± 18	55–62
6	5	400 ± 80	22–33
	2	252 ± 84	28–57
	1	252 ± 36	72–96
10	10	350 ± 70	16–23
	2	338 ± 8	82–87
11	10	250 ± 10	20–22
	5	244 ± 42	34–48

urine decreases as the amount injected increases. These results apply to five out of a series of six rabbits. No relation was found between the total amounts excreted and the weight of the animals.

It follows that within the concentrations quoted the total amounts of antidiuretic hormone excreted in the urine are to a high degree independent of the hormone concentration in the blood.

(*b*) *The time relations of the excretion of the antidiuretic principle.* The urine was collected at intervals of 15 min. after the injection. Upon estimation of the antidiuretic activity of the samples it was found that, with doses ranging from 10 to 1 milliunits per c.c. blood, the bulk of the excreted amount was eliminated within the first 15 min. The second sample obtained between 15 and 30 min. contained only 10–15 p.c. of the total amount excreted. The experimental procedure used does not permit us to decide whether (1) the antidiuretic activity of the second sample is due to a "washing out" of the residue of the pituitrin excreted during the first 15 min., or (2) the antidiuretic principle is really excreted between the 15th and 30th minute after injection, or (3) the antidiuretic hormone content of the second sample is due to both those processes. In no normal rabbit was any antidiuretic activity observed in urine samples obtained later than 30 min. after the intravenous injection.

The possibility that any significant amount of the excreted antidiuretic principle was destroyed during the storage of the urine in the bladder was excluded by estimations of the antidiuretic activity of

mixtures of pituitrin and urine before and after incubation at 39° C. for 5 hours.

(c) *The excretion of the antidiuretic principle in a rabbit with nephritic changes of the kidneys.* It was mentioned above that divergent results had been obtained with one of the rabbits used in the excretion experiments (see p. 89). Two experiments were performed with this animal (No. 8): 10 and 5 milliunits pituitrin respectively per c.c. blood were injected intravenously and the excretion of the hormone determined in the usual way. The animal differed from the other five of the series in the following points. (1) The total amount of hormone excreted was considerably higher, i.e. 700–1000 milliunits as compared with the 160–480 milliunits of the "normal" animals. (2) The excretion of active hormone extended into the 2nd hour after the injection, whereas the normal animals excreted the antidiuretic principle within 30 min. (3) The water diuresis was not inhibited. Normal animals after the intravenous injection of 425 and 850 milliunits per kg. rabbit excreted 3–10 c.c. urine during the 1st hour. For the same period the figures obtained with animal No. 8 were 35 and 21 c.c. of urine.

In connexion with the last point it will be remembered that a diminished sensitivity to the antidiuretic hormone of the pituitary has been thought to be present in cases of human nephritis [Lebermann, 1929; Hart, 1929]. It was, therefore, investigated whether the abnormal behaviour of rabbit No. 8 was coincident with diseased kidneys.

At the time when the above-mentioned experiments on this rabbit were performed only traces of protein were present in the urine. Such traces, i.e. amounts below 20 mg./100 c.c., are not uncommonly found in the urine of rabbits and are hardly of any pathological significance. Four weeks after the experiments, however, a definite proteinuria was present in rabbit No. 8 (130 mg./100 c.c. in the urine collected during 24 hours). This increase in the amount of protein excreted was thought to be significant as it could not be demonstrated in any of the other animals of the same series, some of which had been injected with large doses of pituitrin even more frequently. Animal No. 8 was finally killed and sections of the kidneys were examined. The stains used were hæmatoxylin and eosin and Weigert's iron hæmatoxylin and van Gieson. Dr G. R. Cameron had the kindness to investigate the sections for pathological changes and gives the following report:

"Scattered through an otherwise normal kidney are glomeruli in various stages of degeneration. Some show distortion of shape, are more cellular and contain an occasional polymorphonuclear leucocyte and

lymphocyte. Others are greatly contracted, being reduced to about one quarter the normal diameter. In these the basement membrane lining the endothelial cells of the tufts are adherent to the capsule. No actual cell crescents however are present. The convoluted tubules appear swollen in places, but there is no obvious pathological alteration of their cells. Blood vessels are normal. The condition suggests a mild degree of subacute local glomerulo-nephritis."

The renal action of "bound" antidiuretic hormone

It has been shown in the first two sections of this paper that the antidiuretic principle up to concentrations of about 10 milliunits per c.c. is adsorbed by some colloidal constituent or constituents of the blood. This adsorption compound when injected subcutaneously is not, or very slowly, absorbed from the subcutis and, therefore, has no appreciable action on the kidney. When brought in contact with the kidney by intravenous injection, however, the adsorption compound seems to have the renal action of the "free" hormone.

Rabbits with a satisfactory diuretic response to a given quantity of water were selected. A few c.c. of blood were obtained from the ear vein of the unanæsthetized animal and either defibrinated or mixed with sodium citrate. An hour later 2 p.c. of the body weight of water were given by stomach tube and the animal was placed in a metabolism cage where it remained for 3 hours. A second dose of water amounting to 4 p.c. of the body weight was then given. Finally, 45 min. after the second administration of water, a mixture of 2–4 c.c. of the rabbit's own blood with pituitrin or the same amount of pituitrin diluted with saline was injected intravenously. The urine was collected every 30 min. The activity of the saline-pituitrin and blood-pituitrin mixtures was assessed by determinations of the urine volume and the characteristic increase in the elimination of chlorides. Blood alone had no influence on either the water diuresis or output of chlorides. The doses of pituitrin injected varied from 8 to 0·7 milliunits per kg. rabbit. The concentration of antidiuretic hormone in the pituitrin-blood mixtures was either 10 or 5 milliunits per c.c. blood. Little or no difference could be found between the action of the pituitrin-saline and the pituitrin-blood mixtures as measured by the degree of inhibition of water diuresis and the increase in chloride elimination (Fig. 2). Similar results were obtained in five other experiments. Diuresis experiments with rabbits do not admit the same accuracy in interpretation as results obtained with dogs or rats. We hesitate, there-

fore, to suggest that the results of the injections with bound and free pituitrin are quantitatively identical.

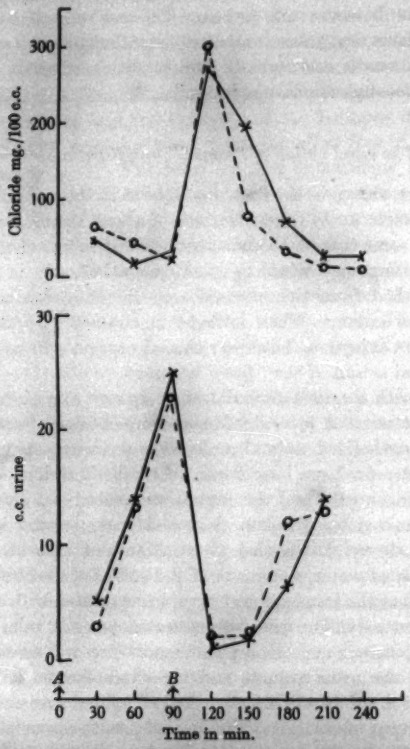

Fig. 2. Rabbit no. 15, ♂, 2300 g. The action of an intravenously injected pituitrin-blood mixture on water diuresis and urinary chlorides as compared with the action of the same amount of pituitrin dissolved in saline. Four c.c. of the rabbit's own defibrinated blood containing 20 milliunits pituitrin, and 4 c.c. normal saline containing the same amount respectively were injected. Continuous line = pituitrin + saline; broken line = pituitrin + blood. *A* = water by stomach tube; *B* = intravenous injection.

There seem to be two possibilities which may explain the renal action of the adsorbed antidiuretic principle. The hormone can be (1) adsorbed

without involvement of its active groups. It is conceivable that in this case the adsorption compound could still have a renal action if brought in contact with the renal cells while its absorption after subcutaneous injection would be prevented by its large molecular weight. The antidiuretic principle could (2) be adsorbed with involvement of its active groups. Under these circumstances a renal action of the intravenously injected hormone could only occur after decomposition of the adsorption compound, i.e. after liberation of the hormone in the kidney. In view of the fact that such a liberation of the antidiuretic principle has actually been demonstrated in the excretion experiments of the preceding section, the second possibility seems the likelier explanation.

Discussion

Mixing of the antidiuretic principle of posterior pituitary extract (pituitrin) with blood *in vitro* results in the formation of an adsorption compound between the hormone and some colloidal constituent or constituents of the blood. This could be demonstrated by the loss of renal activity of blood-pituitrin mixtures after subcutaneous injections into rats, the large molecular weight of the adsorption compound preventing its absorption. The "bound" state of the antidiuretic principle in mixtures with blood could, secondly, be shown by the observation that ultrafilter membranes which are permeable to "free" pituitrin become impermeable to the hormone when it is in the adsorbed state. The adsorbing capacity of the blood for the antidiuretic hormone is limited but constant within the limits of our method for the same species of animal. For the rabbit it was found to be in the neighbourhood of 10 milliunits per c.c. blood. There is strong evidence that the formation of an adsorption compound occurs *in vivo* as well as *in vitro*. An important part of this evidence is furnished by the finding that the activity of suitably small doses of intravenously injected pituitrin, as measured by the rat method, disappears from the blood more rapidly than can be explained by either elimination by the kidney, destruction in the blood or absorption into the tissues.

When small amounts of pituitrin are intravenously injected into rabbits a large proportion is excreted in the urine in a free state. If larger amounts of the hormone are injected the amount excreted in the urine is not proportionately increased. Thus as the amount of hormone injected is increased, the proportion of the hormone which is excreted in the urine decreases (see Table IV). The above observations are based on experiments in which the amounts of antidiuretic hormone injected

have ranged from 1 to 10 milliunits per c.c. blood. They are not likely to apply to amounts of pituitary hormone which are outside this range.

It is evident from the foregoing results that a part of the adsorbed antidiuretic hormone is excreted in the urine in a free state. It is, therefore, concluded that the adsorbed antidiuretic hormone is liberated in the kidney; in other words that the kidney possesses the faculty to elute the adsorbed hormone from its colloidal carrier.

There seems to be no other instance of a similar excretory mechanism for a physiologically active non-foreign substance, but an interesting parallel can be found in the urinary elimination of certain organic dyes, especially phenol red. These seem to be adsorbed by the colloids of the blood and liberated by the kidney in much the same manner as the antidiuretic hormone of the pituitary gland [Marshall & Vickers, 1923; Marshall & Crane, 1924; Marshall, 1931; Grollman, 1925; Bennhold, 1932; Shannon, 1935; Goldring *et al.* 1936].

Since an abundance of evidence indicates [Wearn & Richards, 1924; Richards & Walker, 1930; White, 1932] that the normal glomerulus is only permeable for ultrafiltrable substances, it seems justifiable to assume that non-ultrafiltrable substances like "bound" phenol red or "bound" antidiuretic hormone are excreted by the tubules. In the case of phenol red this is strongly suggested by the work of Marshall [1931], Richards & Walker [1930], Chambers & Kempton [1933], Shannon [1935] and Goldring *et al.* [1936].

The physiological significance of the adsorption of the antidiuretic hormone may be, that small amounts of hormone circulating in the blood are thereby able to avoid elimination by the glomeruli and are automatically guided to the tubules, the probable site of their renal action.

Summary

1. The antidiuretic principle of posterior pituitary extracts (pituitrin) is adsorbed by some colloidal constituent or constituents of the rabbit's blood to the extent of about 10 milliunits per c.c. blood. Evidence is presented which leads to the conclusion that the adsorption of the antidiuretic hormone takes place *in vivo* as well as *in vitro*.

2. The antidiuretic principle dissolved in saline passes rapidly through a collodion ultrafilter, but the ultrafiltrate of a mixture of rabbit's blood with the hormone in concentrations up to 10 milliunits displays no appreciable antidiuretic activity.

3. If antidiuretic hormone is injected intravenously into rabbits a fraction of the injected amount is excreted in the urine. Within a certain

range (1–10 milliunits of pituitrin per c.c. blood) the amount of antidiuretic hormone excreted by the kidney is highly independent of the hormone concentration in the blood. The proportion of antidiuretic hormone excreted in the urine decreases as the amount injected is increased. The urinary excretion of the antidiuretic principle by the rabbit ceases approximately 30 min. after the intravenous injection.

4. These observations applied consistently to five out of a series of six rabbits. The sixth or "abnormal" animal was found to suffer from glomerulo-nephritis.

5. The kidney possesses the faculty to liberate the adsorbed antidiuretic principle.

6. It is suggested that small amounts of the antidiuretic hormone circulating in the blood are thus liberated and eliminated by the tubules.

I wish to offer my sincere thanks to Prof. F. H. Smirk for a valuable suggestion.

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THE VAGINAL HYDROGEN-ION CONCENTRATION IN MONKEYS INJECTED WITH ŒSTRONE

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(*Received* 22 *October* 1936)

The hydrogen-ion concentration of human vaginal fluid has been studied by several investigators, usually from the point of view of the influence fluctuations in *p*H have on the survival of spermatozoa introduced into the vagina. More recently the question has attracted attention because of the belief that cures which have been obtained in the treatment of gonococcal vaginitis in children with œstrogenic substances may be primarily due, at least in part, to the induction of an acid vaginal reaction [Hall & Lewis, 1936]. The routine injection of a group of bilaterally gonadectomized rhesus macaques (*M. mulatta*) with œstrone, for the purpose of studying certain problems relating to the uterus, provided us with the opportunity of obtaining information about the effect such injections have on the vaginal *p*H. Thirty-five observations were made on fourteen monkeys, which were being variously injected with different amounts of the hormone. Vaginal fluid, diluted with sterile normal saline, was obtained by means of a glass pipette, the saline and the vaginal "secretion" being thoroughly and repeatedly mixed. About 5 c.c. of the mixture were obtained for each estimation, which was made by the glass electrode method. The results obtained are given in Table I.

Conclusion and summary

Our data suggest that the vaginal *p*H, which falls between 5·2 and 8·7, does not vary in any obvious way in relation to the length of time an animal has been under the influence of œstrone, or to the amount of hormone given. On the other hand, there is some indication that the vaginal *p*H of individual animals remains fairly constant under different

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TABLE I. Vaginal *p*H of rhesus monkeys injected with œstrone

Animal and observation no.	Remarks	Total œstrone injected (I.U.)	Days injected	Date of *p*H determination (no. of days from 1st injection)	*p*H
71	92 days since castration	—	—	—	6·8
55.1	57 days since last œstrin injection	—	—	—	7·4
55.2	69 days since „ „ „	—	—	—	8·2
	Bleeding from uterus:				
45.8.1	4 days after injections	15,200	40	44	6·4
3.11	5 days „ „	30,400	40	45	6·1
74.5	7 days „ „	32,200	40	47	5·2
	Latent interval following injections before uterine bleeding:				
75.8	1 day following injections	1,400	14	15	8·4
97.9.3	1 day „ „	14,000	14	15	6·7
5.9.1	1 day „ „	46,000	14	15	7·4
5.9.2	7 days „ „	46,000	14	21	7·2
70.3.2	8 days „ „	27,000	14	22	5·9
97.9.4	12 days „ „	14,000	14	26	7·5
92.6.2	1 day „ „	21,500	40	41	7·4
64.2.2	1 day „ „	22,000	40	41	8·6
95.4	4 days „ „	18,000	40	44	6·4
3.13	Under injection	3,000	4	4	7·1
97.9.1	„ „	4,000	4	4	6·7
95.5.1	„ „	1,800	5	5	7·3
74.6	„ „	3,500	7	7	5·7
3.12.1	„ „	10,000	8	8	7·0
70.4	„ „	24,000	8	8	5·9
97.9.2	„ „	9,000	9	9	7·1
70.3.1	„ „	12,500	9	9	5·7
95.5.2	„ „	7,000	11	11	6·3
64.3	„ „	12,000	12	12	8·7
92.7	„ „	12,000	12	12	8·1
3.12.2	„ „	27,000	14	14	6·3
95.5.3	„ „	11,500	22	22	6·3
45.8.2	„ „	14,400	36	36	6·3
92.6.1	„ „	19,500	36	36	7·3
64.2.1	„ „	20,000	36	36	8·5
78	„ „	7,600	76	76	6·1
98.1	„ „	163,000	163	163	6·6
98.2	„ „	175,000	175	175	6·8
98.3	„ „	180,000	180	180	7·4

TABLE II. The different *p*H figures found on different occasions in each of the animals on which more than one observation was made

Animal no.		Animal no.	
3	6·1, 7·1, 7·0, 6·3	74	5·2, 5·7
5	7·4, 7·2	92	7·4, 8·1, 7·3
45	6·4, 6·3	95	6·4, 7·3, 6·3, 6·3
55	7·4, 8·2	97	6·7, 7·5, 6·7, 7·1
64	8·6, 8·7, 8·5	98	6·6, 6·8, 7·4
70	5·9, 5·9, 5·7		

conditions of stimulation with œstrone (see Table II). These results are in general harmony with those of Chen Mai & van Dyke [1934] on the normal rhesus monkey. Using a similar method of estimation to ours, these workers found that the vaginal *p*H of the rhesus monkey undergoing normal menstrual cycles shows considerable variations, there being no clear evidence of a cyclic change, except perhaps a slight tendency for the *p*H to rise about the time of menstruation. Our results do not agree with those of Hall & Lewis, whose observations on three anæsthetized rhesus monkeys that had been injected with œstrone were made by means of a glass electrode directly introduced into the vagina. According to Hall & Lewis, œstrone induces an acid vaginal reaction. They also suggest that their method of estimation "eliminates possible errors inherent in methods heretofore used in obtaining the *p*H of vaginal fluids". Until the nature of these errors is defined, it is advisable to reserve judgement on the three observations they report.

We wish to express our indebtedness to Dr J. R. Baker for the technical advice he kindly gave us, and to Prof. Robinson for allowing us to use apparatus in his department. The monkeys used in this study were purchased with the aid of a grant to one of us (S. Z.) from the Medical Research Council; the œstrone was generously provided by the Organon Company.

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THE TRANSMISSION OF IMPULSES THROUGH THE CILIARY GANGLION

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The transmission of impulses through the ganglia of the sympathetic chain has recently been studied by Bishop & Heinbecker [1932], Brown [1934], Fischer & Löwenbach [1933], Bronk *et al.* [1935], Eccles [1935 *a*, *b*, *c*], and Obrador & Odoriz [1936]. Of the parasympathetic system, the ciliary ganglion has been chosen for study as it is accessible, compact and comparatively large.

Langley & Anderson [1892] inferred from the paralysis produced by nicotine that the ganglion is a relay station in the pupil constrictor tract. This was confirmed by the investigation of the effect of asphyxia by Langendorff [1894], and by the degeneration experiments of Apolant [1896].

Accounts of the anatomy of the ganglion and its roots have been given by Jegorow [1886] and Christensen [1936]. These authors are agreed that in the cat the ciliary ganglion has a motor root, but no macroscopic sensory or sympathetic roots. However, some sensory fibres reach the ganglion from the nasociliary nerve via a communicating branch to the lateral short ciliary nerve. These fibres form Jegorow's "long root". Attempts to demonstrate a sympathetic root microscopically have been unsuccessful [Apolant, 1896; Christensen, 1936], although chromatolytic changes in the ciliary ganglion after removal of the superior cervical ganglion have been described [Fritz, 1899]. It has been suggested by Mazzantini [1927] and by Müller [1931] that the ganglion acts as co-ordinating centre for pupil dilator and constrictor impulses.

The earlier work on the histology of the ganglion is reviewed by Müller & Dahl [1910]. Its structure has been reinvestigated by Beauvieux & Dupas [1926], Pines [1927], Pines & Friedman [1929] and Slavich [1932]. Pines reaffirms that the ciliary ganglion of

the cat consists exclusively of cells of the sympathetic type, but in the ciliary ganglion of the monkey he finds in addition some cells of the afferent type. This is not supported by Anderson [1905], who concluded from degeneration experiments that there are no cell bodies of afferent type in the ganglion of the cat or of the monkey.

An attempt has been made to study transmission through the ganglion by recording the action potentials set up by stimulating the IIIrd nerve and to examine the relation of ganglion cells to afferent and to sympathetic fibres.

Methods

Action potentials were recorded from the ciliary ganglion and its branches in thirty cats and two *Callothrix* monkeys. The animals were decerebrated by the trephine method under ether, and were kept in good condition for 12 hours. Occasionally nembutal was used to stop reflex movements, as it did not appear to have any effect on the ganglion. The IIIrd and Vth nerves were cut close to the brain. A free exposure of the ganglion was obtained by removal of the left side of the skull in front of the tentorium cerebelli. An approach from above was preferred to the lateral one used by Cooper & Groot [1920], as it avoided wounding the ophthalmic artery, which in the cat arises from the external carotid artery. The orbital capsule was opened over the rectus superior after the ethmoidal vessels had been tied and cut. The rectus superior was excised and the lateral short ciliary nerve was picked up on the optic nerve and followed to the ganglion. The ciliary nerves were dissected off the optic nerve, which was cut distally to permit the removal of the eyeball. The three recti muscles were fixed to the margins of the orbit and their motor nerves crushed. Care was taken to preserve the ganglion's blood supply.

The two monkeys were hemidecerebrated under ether after wide removal of the skull. Nembutal was given intraperitoneally after they had recovered from shock. The monkey's ophthalmic artery arises from the internal carotid, and the approach to the ganglion is comparatively easy.

The leads, stimulating coils, amplifier, oscillograph and camera were of the types described by Eccles [1935*a*]. With a rectilinear input to the amplifier, the recorded potential fell to half its initial value in 1·4 sec. Local screening of the induction coils, the animal, the first stage of the amplifier and all leads was made as complete as possible.

At the end of the experiment the ganglion and its branches were stained in osmic acid and sectioned.

Results

Anatomy

The results of forty-five dissections of the orbit are in close agreement with the account given by Christensen [1936]. Briefly, from the ganglion arise a medial and a lateral short ciliary nerve (Fig. 1). The latter, which is much the larger, receives a constant branch from the nasociliary nerve at a point 5–7 mm. from the ganglion. At this point there is usually an

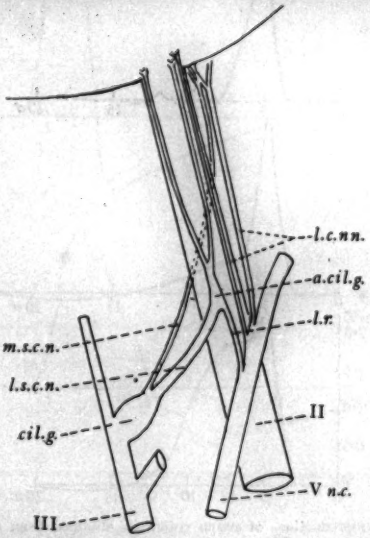

Fig. 1. The connexions of the ciliary ganglion in the cat. *cil.g.*, ciliary ganglion; *l.s.c.n.*, lateral short ciliary nerve; *m.s.c.n.*, medial short ciliary nerve; *l.r.*, the "long root" of Jegorow; *l.c.nn.*, long ciliary nerves; *V n.c.*, nasociliary branch of the Vth nerve. *a.cil.g.* accessory ciliary ganglion.

accessory ganglion, and just distally the nerve breaks up into two or more branches. The medial short ciliary nerve runs direct to the posterior pole of the eye.

Experimental

Fig. 2 shows the action potentials obtained from different preparations by stimulating the intracranial portion of the IIIrd nerve maximally, and leading from the lateral short ciliary nerve just distal to the ganglion. As

usual, approximately monophasic records are obtained by crushing the postganglionic trunk under the distal (grid) lead. Reversing the leads so as to stimulate the short ciliary nerve and to lead from the IIIrd nerve abolishes the action potential, as does painting the ganglion with nicotine. It follows that fibres running from the IIIrd nerve straight through the

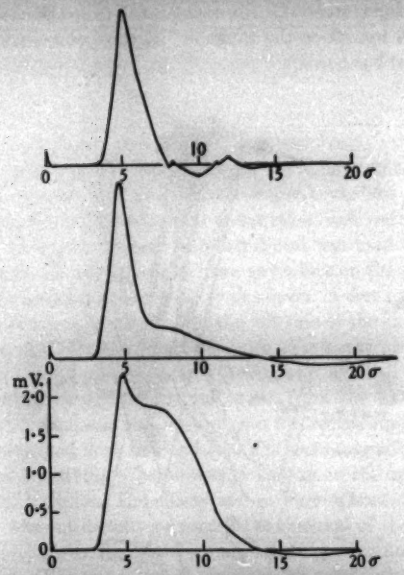

Fig. 2. Enlarged reproductions of action potentials obtained from three preparations. Maximal stimuli, earth leads just postganglionic. The stimulus artefact is shown at zero time. Above, a T_2 spike with a trace of T_n spikes. Centre and below, the T_1 spike is followed by "humps" due to T_n spikes.

ganglion without a synapse are too few to be detected. With the earth lead on the ganglion itself and the whole of the postganglionic trunk isolated from surrounding tissues, the action potential consists of a negative spike (earth relative to grid) beginning after 4σ and lasting $5–10\sigma$, and a slow positive wave reaching its maximum after $40–50\sigma$, and passing off after $125–150\sigma$ (Fig. 5). This slow wave is only obtained with a ganglionic lead, and will be discussed later.

As is shown in Fig. 2, the shape of the spike produced by maximal

stimuli varies from one experiment to another. In all cases there is a sharp initial spike (T_1) which may or may not show a hump on its descending limb. By using submaximal stimuli, or by postganglionic leading, this hump can be made to break up into a number of separate

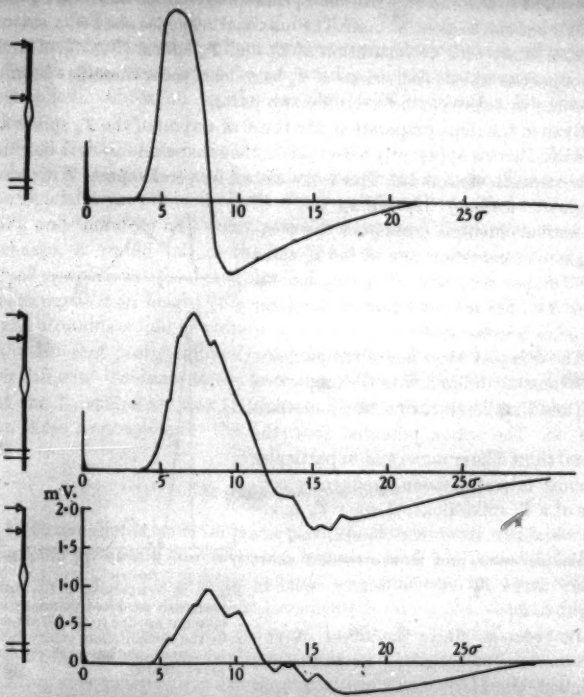

Fig. 3. Above: the response to a maximal stimulus (earth lead close to ganglion). The rounding off of the spike is due to fusion of T_1 and T_n spikes. Centre: the response to the same stimulus, with the earth lead on the postganglionic trunk. Separate spikes have appeared, and the duration of the spike is greater. Below: the response to a submaximal stimulus (leads again postganglionic). The dipharic artefact has not been completely eliminated.

spikes, while the T_1 spike as a rule remains single. Only the first three of these late spikes can be distinguished as T_2, T_3, T_4, and as they all behave alike they will be known as the T_n spikes.

At the ganglion the T_1 spike is seen at 3–5σ after the stimulus (crest times), depending on the length of the preganglionic pathway and the condition of the ganglion. It has a lower threshold, a higher rate of post-ganglionic conduction and, as a rule, a greater amplitude than any of the subsequent spikes. In one case the T_1 spike was smaller than the T_2 spike with all but the weakest stimuli. The maximal response showed a smooth rounded crest, with no separation of T_1 and T_n spikes (Fig. 3). On the two occasions when subdivisions of T_1 have been seen, there has been no difference in behaviour between the two parts.

Even in the same preparation, the times of arrival of the T_n spikes are variable. There is apparently a decrease in the synaptic delay with increase in the strength of stimulus. This is not shown by the T_1 spike. With post-ganglionic leading, "spreading out" of the action potential occurs, as the post-ganglionic conduction rate of the T_1 spike is 5–6 m. per sec., that of the T_2 spike is about 2 m. per sec., and that of the later T_n spikes is even slower.

The different branches of the ganglion show constant differences in the proportion of T_1 and T_n spikes in their action potentials (Fig. 4). The action potential from the lateral short ciliary nerve and in particular its most lateral division, frequently consists of a T_1 spike alone. Larger T_n spikes are obtained from the lateral nerve's medial division, and in the medial short ciliary nerve T_2 may be nearly equal in height to T_1.

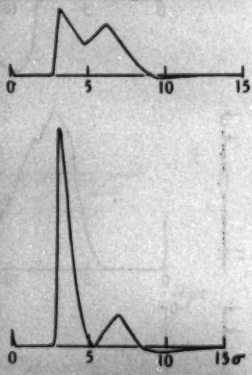

Fig. 4. A comparison of the action potentials produced by a maximal stimulus applied to the IIIrd nerve in the medial short ciliary nerve (above) and the lateral short ciliary nerve (below).

In order to study the effect of two simultaneous preganglionic stimuli on the ganglion, the IIIrd nerve has been split longitudinally and the two parts stimulated independently. With maximal stimuli, the T_1 response to simultaneous stimulation of both halves was found to be equal to the sum of the potentials produced by stimulating each half independently. This must be due to the absence of overlap between the fields of distribution of the two halves of the nerve, unless facilitation can occur. With two successive stimuli applied to the same nerve there is no sign of facilitation in the T_1 spikes, and if such a process exists, it must be over before the end of the relative refractory

period, i.e. 10–12σ, in order to escape detection. Absence of overlap between the two fields of distribution seems the more likely.

With submaximal stimuli to each of the split halves of the nerve, the T_n response to stimulation of both halves together was greater than the sum of the responses to separate stimulation. Evidently facilitation was present. Further, at intervals of 30–100σ, a strong stimulus applied to one half diminished the T_n response to stimulation of the other half. It follows that there must be an overlap in the distribution of the preganglionic fibres on to the ganglion cells responsible for the T_n spikes.

There is little support for the view that the T_n spikes are due to after-discharge in the fibres which have given rise to the T_1 spike. There are no internuncial neurones in the cat's ciliary ganglion [Pines & Friedman, 1929], and therefore no "delay paths". Were the T_2 spike due to after-discharge, it would fall in the relative refractory period due to T_1. Actually, with a maximal stimulus, a T_2 spike 2·5σ after the T_1 spike may almost equal it in height (Fig. 4), at a time when a T_1 spike due to a second stimulus would be reduced to a third of its normal height. In view of the different postganglionic conduction rates of T_1 and T_n spikes, and their different incidence in the branches of the ganglion, it is more likely that the T_n spikes are due to separate fibres with longer time relations than those responsible for the T_1 spike.

The ganglionic slow wave

With ganglionic leads there is a positive after-potential which reaches its maximum about 40σ after the stimulus, and has passed off after 125–150σ (Fig. 5). The greatest potential seen amounted to 30 p.c. of that of the spike. It is conducted decrementally along the postganglionic trunk and disappears after 2–3 mm. In Fig. 5 T_1 and T_n spikes are present, but a slow wave is also set up by a T_1 wave alone. The corresponding slow positive wave in the superior cervical ganglion is larger and of longer duration, reaches its maximum at 100σ and passes off at about 500σ [Eccles, 1935*c*]. After the ganglion has been painted with a solution of 0·002 p.c. nicotine, or with 0·001 p.c. curare, the slow positive wave is slightly smaller, and its maximum is reached after 20σ, i.e. 20σ earlier than before. The slow wave is completely abolished by painting with 0·005 p.c. nicotine, but a solution of 0·1 p.c. is necessary to remove the spike as well. In the superior cervical ganglion this earlier maximum appears to be due to the removal of a small negative wave which normally

masks the beginning of the slow positive wave. In the superior cervical ganglion, both N and P waves have been shown to begin shortly after the end of the spike, but the N wave is over after 50σ. As a result there is a

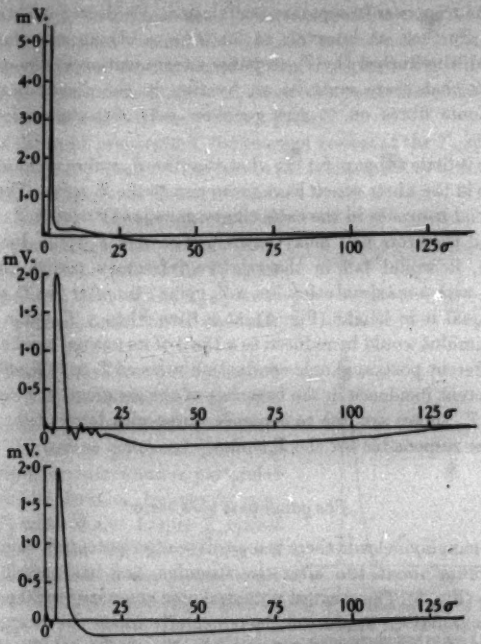

Fig. 5. The ganglionic slow waves. Above: the ganglionic response to a maximal stimulus in the monkey, showing the short duration of the action potential and the negative and positive waves. Centre: the response obtained under the same conditions from the ciliary ganglion of the cat. Below: the response to the same stimulus after the ganglion had been painted with 0·002 p.c. nicotine.

small negative wave followed by a period of increasing positivity [Eccles, 1935*c*]. In the ciliary ganglion of the monkey both negative and positive slow waves are present (Fig. 5), and from the above evidence it is probable that a small negative wave is also set up in the cat's ciliary ganglion.

Facilitation and inhibition

Fig. 6 shows the effect of a maximal stimulus upon the response to a submaximal stimulus set up more than 30σ later. In the second response, the T_1 spike is unaffected although the T_n spikes show obvious inhibition. This is evident at intervals greater than 30σ and passes off at 250–300σ.

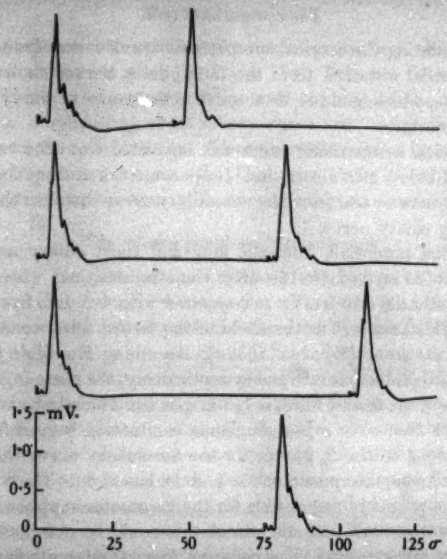

Fig. 6. Inhibition of a response to a submaximal stimulus by another of the same size. Above: three action potentials in which inhibition of the T_n spikes has occurred. Below: the uninhibited response to the second stimulus. In this preparation the T_1 spike, which is not inhibited, shows a small spike on its ascending limb.

Usually all the T_n spikes are inhibited, but T_2 or T_3 may be affected much more than the rest. The depth of inhibition produced and the rate of its development depend on the strength of the first stimulus.

While inhibition has been obvious, facilitation has been slight even under favourable conditions. It can be demonstrated in cases where the T_1 and T_n spikes are well separated, by using small equal stimuli at intervals of 10–20σ, and is limited to the T_n spikes.

As in the superior cervical ganglion, responses which were previously facilitated are inhibited after application to the ganglion of 0·002 p.c. nicotine. It is therefore probable that the inhibitory process in the ciliary ganglion also begins shortly after the appearance of the spike, and is at first masked by concomitant facilitation, as in the superior cervical ganglion [Eccles, 1935*b*].

The sympathetic root

On stimulating the cervical sympathetic trunk it was found that the action potential obtained from the long ciliary nerves showed a well-defined spike, which could be used to trace the course of the sympathetic fibres of the orbit.

The cervical sympathetic trunk was separated from the vagus nerve and crushed below loop electrodes. Leads were taken from the long and short ciliary nerves, and from the nasociliary nerve distal to the origin of the last long ciliary nerve.

The action potentials from the long and short ciliary nerves were alike. The spike arrived 15–16σ after the stimulus, and when maximal reached a potential of 0·5 mV., as compared with 4–5 mV. from stimulation of the IIIrd nerve. The threshold of the fastest fibres corresponds to that of the S_1 group [Eccles, 1935*a*]. Assuming Eccles's figures for preganglionic conduction rate and synaptic delay, the postganglionic conduction rate of the fastest fibres is 7–8 m. per sec. The latest visible spikes appear at 23–25σ, have a postganglionic conduction rate of 3–4 m. per sec., and belong to the S_2 group. In the nasociliary nerve there are no S_1 fibres, the first spike appears at 25σ and the last at 60σ. The corresponding fibres are probably responsible for the vasomotor supply of the nose.

No response to sympathetic stimulation could be obtained from the medial short ciliary nerve. The response from the lateral nerve is due entirely to sympathetic fibres which reach it from the "long root", and is unaffected by section of the lateral short ciliary nerve between the ganglion and the "long root". With sympathetic stimulation no action potential could be recorded from any fibres which may run back from the "long root" to the ganglion. It was still possible that myelinated sympathetic fibres reach the ganglion by its motor root and end in it. Six experiments were carried out to see if a sympathetic volley interfered with conduction through the ganglion, or with the progress of facilitation or inhibition. No such effect was found. There is therefore no evidence that any sympathetic fibres reach the ciliary ganglion in the cat, and no interaction of parasympathetic and sympathetic impulses occurs there.

The sensory fibres

Apart from that due to fibres of 8–10σ which run in the "long root", join the lateral short ciliary nerve and run on to the eye, no action potential due to sensory fibres has been obtained from the ganglion or from the short ciliary nerves.

Rhythmic discharges

In five preparations a spontaneous discharge has been picked up from the short ciliary nerves after the IIIrd, IVth, Vth and VIth nerves had been cut. This was of nervous origin, since the side of the pupil corresponding to a remaining short ciliary nerve was contracted and only relaxed when the nerve was cut. There is evidence that isolated ganglia may set up impulses spontaneously [Jegorow, 1886; Böshamer, 1925], but the exciting cause here may have been an injured area in the ganglion.

The ciliary ganglion of the monkey

The ciliary ganglion of the *Callothrix* monkey gives off five short ciliary nerves and a small branch to the central artery of the retina.

Fig. 5 shows the action potential obtained by stimulating the IIIrd nerve and leading from the ganglion. Although the preganglionic pathway is not shorter than in the cat, the spike appears 2–2·2σ after the stimulus, as compared with an average time of 4·6σ in the cat. The spike has also a shorter duration than the T_1 spike. All branches show this spike, but one also shows a second spike 10 p.c. of the main spike in height. Values of 5·8 and 6 m. per sec. were obtained for the postganglionic conduction rate. Fig. 5 shows the small ganglionic slow wave, which possesses negative and positive phases. Both are removed by 0·005 p.c. nicotine, and a solution of 0·02 p.c. removes the spike.

No trace of facilitation or inhibition was found at any time. No action potentials were found in the short ciliary nerves after stimulating the cervical sympathetic trunk. A small action potential was found on stimulating one ciliary nerve and leading from another. This was abolished by reversing the leads, or by painting the ganglion with nicotine. The nature of this reflex arc was not determined.

Discussion

The existence of facilitation and inhibition in T_n fibres brings the ciliary ganglion into line with those of the sympathetic chain [Eccles, 1935*b*; Obrador & Odoriz, 1936]. Both processes are, however, much

less obvious than they are in the sympathetic ganglia. On the other hand, the T_1 fibres which form the larger part of the postganglionic outflow show no sign whatever of facilitation or inhibition, and all the postganglionic fibres in the monkey behave in the same way. Their behaviour is that to be expected if each ganglion cell receives endings from a single preganglionic fibre, and the evidence supports this view. No difference in function between T_1 and T_n fibres has been established.

It is not suggested that the ciliary ganglion is in any way typical of parasympathetic ganglia.

Summary

1. A method is described of studying the behaviour of the ciliary ganglion.

2. In the cat two types of fibre have been found. The first (T_1) has a low threshold, short synaptic delay, rapid conduction rate, and shows neither inhibition nor facilitation. The second type is made up of a number of subgroups which have a higher threshold and a slower conduction rate than T_1, and show some facilitation and considerable inhibition.

3. In the monkey, transmission through the ganglion is very fast, and all the cells behave in the same way as the T_1 cells of the cat.

4. In both animals ganglionic slow waves have been seen.

5. A spontaneous rhythmic discharge has been seen on leading from the decentralized ganglion.

6. No support has been obtained for the view that the ganglion is a co-ordination centre for pupil movements.

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ON THE EFFECT OF ALTERNATING CURRENTS ON THE COCHLEA

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Within the last few years we have attempted to use electrical stimulation of the organ of hearing in man as a method of studying its functional properties [Gersuni *et al.* 1934].

We have used single-condenser discharges and induction shocks to determine the chronaxie [Gersuni *et al.* 1935] and the refractory period [Gersuni & Volokhov, 1935] of certain elements of the auditory apparatus, and our analysis shows that these are probably the nerve fibres or the endings of the auditory nerve.

It is more difficult to determine the region affected by alternating currents. The sensations which originated during the flow of such currents through the ear are of a tonal nature and closely resemble in their character those arising from sound stimulation of the same frequency [see also Fromm *et al.* 1933]. The possibility of producing beats between electric and sound vibrations simultaneously conveyed to the same ear also confirms the likeness between the phenomena observed in the case of electric and sound stimulation [Gersuni & Volokhov, 1934, 1936].

This resemblance between the sensations caused by electric and by sound stimulation made it necessary to be extremely cautious in estimating the phenomena observed, since mechanical oscillations might be generated in certain portions of the circuit and might affect the ear in the usual way.

However, we have already reported [Gersuni & Volokhov, 1934, 1936] a number of observations showing that the auditory sensations are due to a direct effect of alternating current on the cochlea and not to vibration of the electrode, of the liquid, or of the skin, etc. A new series of controls has now been made and the view that alternating current has a direct effect on the cochlea has found further confirmation.

Methods

The experiments were carried out on three trained individuals to whose auditory apparatus alternating current of frequencies from 100 to 14,000 c.p.s. were conducted. The source of the electric oscillations was a heterodyne oscillator constructed by the All-Union Institute of Metrology and Standardization in Leningrad. The alternating current passed through the body was measured by means of a vacuum thermocouple. The impedance of the body was measured by means of a bridge, the variable branch of which consisted of a resistance and a capacity connected in parallel.

The electrodes used were of different kinds depending on the conditions of the experiment: (1) a thin silver wire with a bulb-like thickening at the end and encased in rubber tubing 2 mm. in diameter, the edge of the rubber tubing projecting by ½ mm. and thus preventing the metal from coming into direct contact with the skin; (2) a rubber stopper from 5 to 6 mm. in diameter with a thin silver rod passed through the centre and projecting by 2–3 mm. beyond the surface of the cork; (3) a metal rod connected with the armature of a loud-speaker and encased in thin rubber tubing. The latter electrode was simultaneously used as a sound radiator, the arrangement being as follows: the diaphragm and the base of the loud-speaker were removed and the electromagnetic system firmly adjusted on a stand with the stylus directed downwards; the latter was then connected with the electric circuit used for stimulating. This circuit was completely independent of the circuit of the electromagnetic sound-radiating system.

The indifferent electrode was a silver plate having a surface of 15 sq. cm. which was covered with gauze soaked in a 0·9 p.c. normal saline solution.

The subject was connected into circuit in the following manner: he was placed in a horizontal posture and some normal saline solution at body temperature was poured into his external auditory meatus; an electrode of one of the types described above was then introduced into the liquid without touching the walls of the auditory meatus.

The determination of thresholds both in the case of electric and of sound (i.e adequate) stimulation was effected by means of intermittent stimulating periods each lasting no longer than 0·5–1 sec. This, in the first place, facilitated the estimation of thresholds for the subject and, secondly, reduced to a minimum the possibility of the auditory threshold being affected by fatigue to sound.

The sound vibrations were conveyed to the ear by the loud-speaker the stylus of which was immersed in the liquid in the auditory meatus (electrode 3). The changes in the intensity of the sound were effected by an attenuator which was shunted by a resistance of 650 ohms.

With the low electrical intensities at which we were operating, it was possible to accept the existence of a linear relationship between the magnitude of the current and the resultant sound intensity from the sound-radiating system.

The general scheme of the apparatus is shown in Fig. 1.

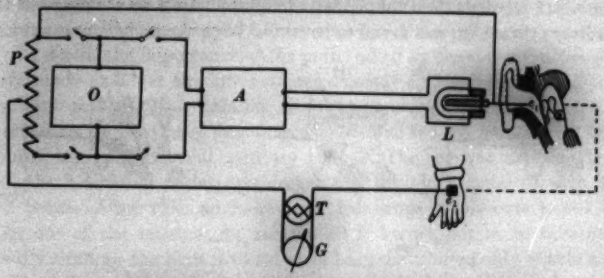

Fig. 1. *P*, potentiometer; *O*, oscillator; *A*, attenuator; *L*, loud-speaker; e_1, e_2, electrodes; *T*, thermocouple; *G*, galvanometer.

Results

(i) Alternating current varying in frequency from 100 to 14,000 c.p.s. flowing through the auditory apparatus was found to give rise to auditory sensations closely resembling those resulting from sound stimulation. It was of interest to find out whether these sensations might not be attributed to the sound radiation occurring near the electrode (vibrations of the stylus itself or of the surface forming the boundary line between the electrode and the liquid). The occurrence of such phenomena was probable in view of the experiments carried out by Kahler & Ruf [1931] who had observed sound vibrations to originate in the boundary surface between the electrode and the dry skin, when alternating current was passed through the body.

Now if mechanical vibrations actually originate near the electrode, then, by increasing acoustic resistance between the electrode and the ear, it should be possible to produce an attenuation of the sound so that an auditory sensation would only arise when considerably stronger currents were used. To see if this occurred we immersed the electrode (the stylus

of the loud-speaker) in a glass tube 225 cm. long and 5 mm. in diameter filled with saline solution. The other end of the tube was inserted in the external auditory meatus which was also filled with saline solution. The tube was cut in half, the two sections being connected by means of rubber tubing and the distance between their edges being 3–4 cm. The rubber tubing was stuffed with absorbent cotton (see Fig. 2, I). The electrical impedance of the entire system was 70,000 ohms for an alternating current of 1000 c.p.s., which was the frequency employed in the experiments. The attenuation in this system when the sound was conveyed through the tube, which manifested itself as a change in the auditory threshold, was found to be 80 db. With the body connected into

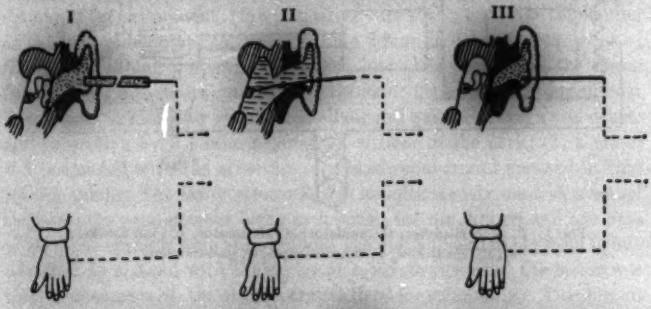

Fig. 2. Saline solution; paraffin oil; non-conducting layer of paraffin oil.

the alternating current circuit, the strength of the current of threshold value was determined both with and without this system of tubes being connected in circuit. All the estimations have failed to show any noticeable changes in the threshold values, which were independent of the acoustic resistance being connected or disconnected.

	Thresholds in mA.	
N	Electrode in the external auditory meatus	Electrode in the tube
1	0·48, 0·50, 0·46	0·37, 0·30, 0·32
2	0·40, 0·42, 0·39	0·28, 0·30, 0·32
3	1·08, 1·1, 1·1	1·0, 1·04, 1·0

Thus the changes developed in the opposite direction to that which might have been expected if the vibrations had originated near the electrode.

This experiment clearly showed that the phenomena observed during the passage of alternating current could by no means all be due to any mechanical processes occurring near the electrode.

(ii) Further experiments were made to decide whether mechanical vibration might occur along the boundary line between the liquid and the skin. If the effect depends on such vibrations, then the paths along which the current flows from the auditory meatus cannot be of any particular importance. The only essential factor would be the existence of contact between the skin and the liquid over a sufficiently large area, for the high sound conductivity of water would ensure that mechanical vibrations would be conveyed from any section of the auditory apparatus.

To decide the importance of the paths taken by the current, attempts were made to cover the lower or more deeply situated portions of the auditory meatus with paraffin oil. Experience proved the following method to be the most practicable one. One c.c. of paraffin oil was poured into the external auditory meatus. The ear was then stopped and the individual under experiment was kept in a lying posture for 2–3 hours. After this, no more than 1–2 drops of oil were found to pour out of the meatus. As paraffin oil is known not to be absorbed readily through the skin it could have been distributed only within the walls of the auditory meatus. After the manipulation had been completed the auditory canal was dried and then filled with saline solution (see Fig. 2, III). The resistance of the circuit of the body was not found to show any significant change; however, when current was passed it failed to give rise to any auditory sensations in one of the individuals, while in the other the thresholds were found to be many times increased.

	Previous to unction with oil		Subsequent to unction with oil	
	Thresholds in mA.	Resistance (ohms)	Thresholds in mA.	Resistance (ohms)
1	0·38, 0·40	2800	No sensations at 4·0	2715
2	0·1, 0·1	2100	3·8, 3·7	1400
3	0·3, 0·18	2270	1·6, 1·7	2000

It was possible to judge of the actual change in the paths taken by the current from the following observations: as a rule an increase in the strength of the current always gave rise to painful sensations which were located in the depths of the auditory meatus. Subjectively, they were perceived as a feeling of pressure or tension and later as a prick somewhere very deep in the auditory meatus. Following the administration of oil these sensations failed to develop; all the painful sensations were located in the upper portions of the auditory canal near the tragus. These facts

could only be explained by assuming a change in the paths taken by the current.

It is of importance to note that a marked increase in the thresholds for electrical stimulation lasted for several (from 3 to 6) days following the administration of oil and was found to wear off gradually. At the same time, it should be observed that no change was found to occur in the auditory thresholds as measured by sound stimulation. All the data reported served to show that a mere contact between the liquid and the skin—which had remained unimpaired in the upper portions of the external auditory duct—was not in itself sufficient to produce any auditory sensations even when currents exceeding tenfold the threshold strength were employed.

(iii) If the effect was due to fluctuations in the volume of the liquid in the external auditory canal, the stopping of the latter with a cork should intensify the pressure changes and therefore the sensation resulting from them. The measurements taken have shown that this phenomenon does not occur. The thresholds failed to reveal any changes when a cork electrode was used; so, for instance, in subject V., the thresholds were 0·3 mA. when the auditory meatus was open and of the same value (i.e. 0·3 mA.) when the canal was stopped up. Where both the electrodes were introduced into the liquid contained in the meatus the auditory sensations either failed to arise or arose only when the strength of the current considerably exceeded the threshold value. For instance, the normal threshold in subject A. was 0·18 mA.; while no sensations were found to arise even at 3·6 mA., when both the electrodes were simultaneously introduced into the fluid; in subject G. no sensations arose at 4·0 mA. when both the electrodes were in the fluid, while under ordinary conditions the threshold was 0·25 mA.

These experiments served to show that there was no reason to assume that vibrations in the column of liquid contained in the external auditory meatus were the cause to which the origin of auditory sensations might be attributed.

(iv) Andreev *et al.* [1935] have shown that the auditory sensations due to alternating current also arose when all the apparatus of the middle ear had been removed.

The fact that the ear drum played no important part in giving rise to auditory sensations when electrical stimulation was used was also confirmed by experiments in which the pressure in the external auditory meatus was changed. The detailed investigations carried out by Békésy [1929] have shown that changes in the pressure on the eardrum may lead

to changes of loudness which are of different values at different stimulation frequencies. In the course of our experiments we have effected an increase of pressure by means of a column of liquid. The pressure was increased by 25–30 mm. of mercury. It was then observed that, though for sound stimulation the perceived loudness underwent marked changes when there was a change of pressure, both the loudness and the nature of the sensations remained unaltered when electric stimulation was used.

(v) Thus, all the experiments cited above bore witness to the fact that no mechanical vibrations originate in any of the systems situated in the cavity of the external or the middle ear. Naturally this led to the conclusion that the alternating current must affect certain systems in the inner ear. This conclusion has been further confirmed by the following experiment: an electrode was immersed in a drop of normal saline solution and then fixed near the promontory in individuals in whom the apparatus of the middle ear had been removed. The entire cavity of the middle and external ear was filled with non-conductive paraffin oil (see Fig. 2, II). By this means the paths taken by the current were confined as far as possible to the highly conductive media of the inner ear. The magnitude of the current passing through the superlying strata was undoubtedly limited. In spite of this, the thresholds observed for electrical stimulation revealed no noticeable changes.

	Saline solution		Paraffin oil	
	Thresholds in mA.	Resistance in ohms	Thresholds in mA.	Resistance in ohms
1	0·30, 0·28	1280	0·25, 0·28	1550
2	0·29, 0·30	1525	0·31, 0·30	1590
3	0·22, 0·23	1645	0·23, 0·25	1420

(vi) On placing an electrode on the dry skin of the mastoid process, Kahler & Ruf [1931] were able to hear, with the help of a stethoscope, sounds arising in various portions of the head on conducting alternating currents through the body. Using the method of procedure we have described above, we have never once observed such a phenomenon. Thus, though the sounds produced by the stylus of a loud-speaker placed in the liquid of the auditory canal could be readily heard in various portions of the head when the sensations had reached a level of about 30 db., the observer failed to hear any sound phenomena arising from alternating current producing equally loud sensation to the subject.

The data reported above may be summarized as follows:

(1) The auditory sensations which arise when alternating current is passed through the auditory apparatus do not result from vibrations of

the electrode or the development of mechanical forces at the boundary surfaces between the liquid and the skin.

(2) The auditory sensations cannot be caused by a change in the volume of the liquid contained in the auditory meatus.

(3) The effect produced by the action of alternating current does not change when the apparatus of the middle ear has been removed or a change in the pressure on the eardrum has been effected.

(4) Alternating current ceases to produce any effect when the spread of current through the inner ear has been limited.

(5) The effect produced by alternating current fails to reveal any changes when the spread of current through the cavity of the external and middle ears has been limited.

All the above data appear to lead to only one possible conclusion; namely, that auditory sensations are found to arise only when alternating current is passed through the internal ear (the cochlea), and not when they pass through any other portion of the auditory apparatus.

This conclusion renders it necessary to suppose that alternating current immediately affects those portions of the inner ear which are connected with the origin of auditory sensations. Therefore, we may consider the cochlea as being the site of action of alternating current [see Gersuni & Volokhov, 1934, 1936].

Naturally, the question arises as to the mechanism of the effect produced by alternating current on the cochlea. This problem will be discussed in our next report [Arapova *et al.* 1937].

Summary

1. The paper describes a method of stimulating the organ of hearing in man by means of alternating current.

2. The sound sensations which are observed to arise when alternating current of sonic frequency is passed through a normal auditory apparatus very closely resemble in their nature those caused by sound stimulation.

3. It can be proved that the cause of these sensations does not lie in any phenomena occurring in the alternating current circuit, such as vibration of the electrode, or of the liquid which surrounds it, or of the skin.

4. The condition necessary to secure the appearance of these sensations is that the alternating current must be passed through the inner ear. Hence it follows that the sensations can only be due to the direct action of alternating current on certain structures within the internal ear (i.e. the cochlea).

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A FURTHER ANALYSIS OF THE ACTION OF ALTERNATING CURRENTS ON THE AUDITORY APPARATUS

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In studying the mechanism of action of alternating current on a normal auditory apparatus, Gersuni & Volokhov [1934, 1936] have obtained a number of experimental data pointing to the fact that the tonal sensations are due to the passage of alternating current through the cochlea. The question which naturally followed was: What elements of the cochlea were affected by the alternating current, and what theoretical mechanism for their effect may be suggested? It seemed obvious that the production of a complete series of tonal sensations corresponding to the range of frequencies 100 and 14,000 cycles per second could not be ascribed to the immediate effect of alternating current on the fibres of the auditory nerve. In addition to the improbability of this assumption from the point of view of the physiological properties of nerve fibre in general, it was contraindicated by a number of data on the effects of electrical stimulation of the auditory apparatus [Andreev *et al.* 1934, 1935].

The following possibilities may be suggested to explain the mechanism of the effect produced by alternating current on the cochlea:

(1) The alternating current may cause alternating mechanical forces to develop in the cochlea. The stimulation of the receptor elements thus occurs in the usual way, i.e. as a result of the action of mechanical forces.

(2) Omitting the stage of transformation from electrical to mechanical forces, alternating current may immediately affect certain receptor (i.e. cellular) elements of the cochlea, and this in turn may lead to the production of an auditory sensation.

The present paper reports (*a*) the data that have been obtained by a study of the rise in the loudness of the sensation which results from increasing the strength of the stimulating current; and (*b*) the data obtained on the pitch of the tones which result from stimulation by alternating current of different frequencies. These data may serve as a foundation for solving the problem of the mechanism of action of alternating current on the cochlea.

Methods

The essential principles of the apparatus used and of the methods employed were described in detail in the preceding paper [Gersuni & Volokhov, 1937].

Both the loudness and the pitch of the tonal sensations produced by electrical stimulation were determined by comparing them with those of equal loudness and pitch produced by sound stimulation. For this purpose, electrical and sound stimuli were applied in turn by means of the electrode and the loud-speaker previously described. In making these comparisons, six signals of definite intensity and frequency were usually given one after the other at intervals of not more than 1 sec., each signal lasting from 0·5 to 1 sec.

The intensity of the sound stimuli was expressed in decibels above the threshold (i.e. sensation level). In studying the magnitude of the sensation during electrical stimulation the strength of the alternating current was adjusted until it caused a sensation equal in loudness to that produced by sound stimulation at a given level. In studying the pitch of the tones the frequency of electrical stimulation was left unchanged and a sound stimulus of frequency such as to cause a sensation of the same pitch was then found.

The experiments were carried out on three trained individuals.

On the pitch of the tonal sensations incurred by electrical stimulation

The pitch of the "tones" occurring during electrical stimulation was determined for a number of frequencies (from 500 to 4000 c.p.s.). The largest number of experiments was carried out with frequencies of 1000 and 2000 c.p.s. It was found that coincidence between the pitch of tones for electrical and adequate (i.e. sound) stimulation was not observed for all the frequencies. As a rule, in subjects A. and V., the tone seemed to be higher when electric stimulation was used, the ratio between the frequencies corresponding to a given pitch being 1 : 2.

However, a frequency at which the pitch of the tones coincided for both kinds of stimulation could be found for each of the individuals subjected to the test, as will be seen from the following data:

TABLE I

	Alternating currents (c.p.s.)	Pitch	Remarks
Subject A.	500	1000	
	750	1500	
	1000	2000	
	2000	2000	
	4000	4000	
Subject V.	500	1000	
	1000	1000	
	1500	3000	Not pure
	2000	4000	
Subject G.	500	500	Not pure; a metallic component is distinguishable
	1000	1000	
	1500	1500	

In subject A. the sensations caused by electrical and sound stimulation at a frequency of 2000 c.p.s. were found to have so close a resemblance that the electrical and the sound stimuli failed to be differentiated. It should be noted that this close resemblance between the sensations caused by either kind of stimulation was only observed at an intensity corresponding to a sensation level of not more than 15–20 db.; indeed, the nature of the sensation produced by electrical stimulation was found to be, in great measure, dependent on the magnitude of the stimulating current. Thus, when currents of more than 1–1·5 mA. were applied, and the sensation level exceeded 20 db., the sound acquired a metallic character and was no longer pure; a further increase in the strength of the current gave rise to certain hissing sounds, after which the sensation finally became vague, increased in volume, and lost its clearly manifest tonality, for different tones could be heard simultaneously. The sensation was then of quite another nature to the equally loud sensation produced by adequate stimulation.

TABLE II

Frequency	Sensation level (db.)	Strength of current (mA.)	Nature of sensations
2000	10	0·65	The pitch and the character of the tones are the same for electrical and for adequate stimulation
2000	15	0·92	A slightly metallic sound is noticed
2000	20	1·28	The same phenomenon is more markedly manifest
2000	25	1·68	A hissing component is distinguishable
2000	28	2·10	The tone produced by electric stimulation is higher than that caused by adequate stimulation

The changes occurring in the nature of the sensation when the strength of the current is increased may be seen from Table II.

Mention should be made of yet another phenomenon: one of the individuals under observation (subject G.) was found to have no sensation, within a definite zone of frequencies, when using electrical stimulation (see Table III), while the thresholds for the same frequencies for adequate stimulation failed to reveal any deviation from the normal.

TABLE III

Frequency	Threshold in mA.	Frequency	Threshold in mA.
500	0·40	2000	No sensations (2·5 mA.)
1000	0·38	3000	—
1500	1·1	5000	1·0

It is of interest to note that, in the case of the other two individuals, the threshold values of the stimulating current within the range of frequencies 1000–4000 c.p.s. were found to be almost identical (from 0·28 to 0·30 mA.).

ON THE INCREASE IN LOUDNESS OBSERVED UNDER CONDITIONS OF ELECTRICAL STIMULATION

By increasing the strength of the stimulating current, the loudness of the sensation may be raised to its highest possible magnitude, corresponding to some 30–40 db. above the threshold. As has been previously mentioned, this is accompanied by a marked change in the nature of the sensation. Fig. 1 shows curves illustrating the rise in the intensity caused by currents of increasing strength. The sensation levels in decibels are marked along the ordinate axis (having been determined at each given sound frequency by means of an attenuator); while the ratio between the strength of the current used (I) and its threshold value (I_0) is marked along the abscissæ.

As will be seen from the data shown in Fig. 1, in subject A. the maximum loudness was reached at about 28 db., while for subject V. the maximum occurs at about 40 db. However, all the curves have the same shape. No great importance should be attached to the difference in the limits of loudness observed in the two individuals under experiment, for it apparently depended on the fact that the threshold values of painful sensations were considerably lower in subject A. than in subject V. The latter had a much higher pain threshold and thus it was possible to apply a stronger current (up to 3 mA.) to subject V., the limit for subject A. being as low as 2 mA.). In subject G. the limit of loudness was also reached at about 25 db.; a further increase being again impossible

owing to the onset of painful sensations. Mention should be made of the great sensibility to pain of the auditory meatus of subject G. This phenomenon, together with the high threshold values for electrical

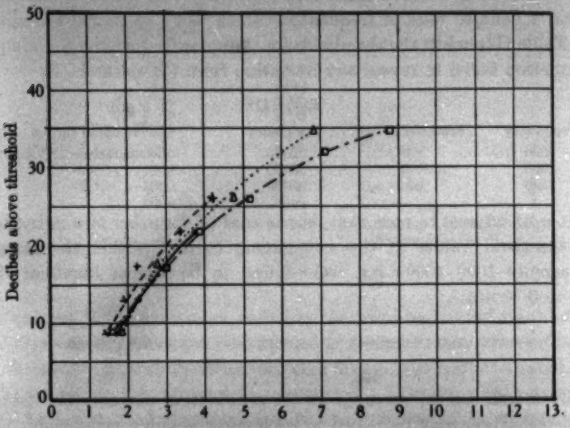

Fig. 1. I/I_0: △···△ 1000 cycles, subject V. □—·—□ 2000 cycles, subject V. ×— —× 2000 cycles, subject A. •—• 1000 cycles, subject A.

stimulation in this subject, made the loudness estimation for a supraliminal current insufficiently accurate. No very definite idea can be formed with regard to the absolute limit of loudness which might be revealed when using electrical stimulation. However, the shape of the curves, which incline towards the abscissæ, as well as the disintegration of the tonal sensation at levels exceeding 30 db., serves to indicate that further increase of the current would fail to produce a rise in the loudness of the sensation beyond a level of 40–45 db.

Fig. 2 shows the mean curve obtained in the course of all the experiments carried out on the two subjects A. and V. at frequencies of 1000 and 2000 c.p.s.

If the values of the strength of the current corresponding to different levels (Fig. 2) be expressed in logarithmic units, we have a linear relation between the sensation level (i.e. the logarithm of the ratio of sound pressures[1]) and the logarithm of the current necessary to produce a

[1] The sensation level in decibels (N) can be expressed as $N = 20 \log P/P_0$, where P_0 is the threshold sound pressure and P is the sound pressure at the given level.

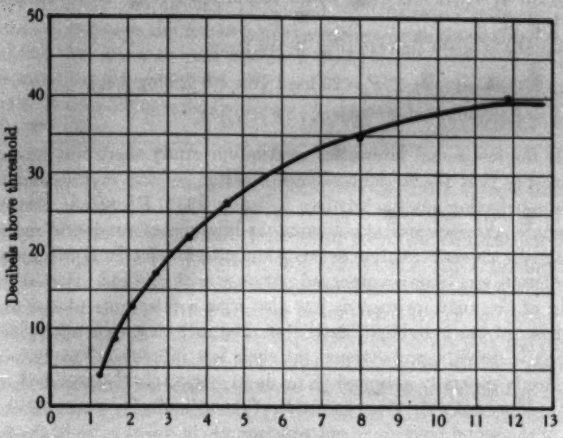

Fig. 2. I/I_0.

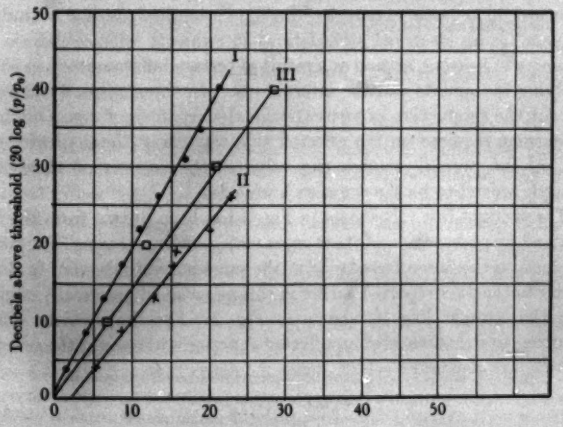

Fig. 3. $20 \log I/I_0$: •—• normal auditory apparatus; ×—× affected auditory apparatus. $20 \log v/v_0$: □—□ cochlear potentials (after Davis *et al.*).

sensation of equal loudness, under conditions of electrical stimulation (see Fig. 3).

For the straight line (*I*) (Fig. 3) we have

$$N = 20 \log P/P_0 = 20 \log 1{\cdot}18 \div 1{\cdot}8 \,[20 \log I/I_0],$$

hence

$$P/P_0 = 1{\cdot}18\,(I/I_0)^{1{\cdot}8}. \qquad \ldots\ldots(1)$$

At the low sound intensities used in our study there was no reason to suppose that the sound-conducting system of the middle and inner ear was behaving non-linearly [see Békésy, 1934; Fletcher, 1930]. At these intensities we may also assume the linearity of our sound-radiating system, for the same relation between loudness for electrical and adequate stimulation was likewise observed not only with the rod inserted in the liquid of the auditory meatus but also with a telephone placed on the other ear of the individual under test. The fact that in a telephone the current is directly proportional (at such low intensities) to the sound pressure is generally accepted [Knudsen, 1923; Geffcken & Heisig, 1934]. If so, not only the magnitude of the stimulating current but also that of the sound pressure in our equation are in direct ratio to the forces applied to the internal ear. Therefore, it is highly probable that the constants of the equation are dependent on the state of such structures of the cochlea as are affected by alternating current. This suggestion is confirmed to a large extent by the data obtained during a study of loudness (using electrical stimulation) in subjects with unilateral disturbances of hearing, arising as a result of certain inflammatory processes involving the middle and the internal ear.[1] In these cases it was found that for the production of supraliminal sensations of equal loudness, the current required on the affected side was many times greater than that on the normal, though the value of the current of threshold strength proved to be the same on both sides.

The straight line (II) shown in Fig. 3 has been drawn from the data obtained by observation of these cases with deficient hearing. The results obtained on the non-affected side in the same individuals were so closely similar to the data reported earlier in this paper as to practically coincide with the straight line (I) shown in Fig. 3. For the straight line II (referring to the cases with an affected function of hearing) the constant $a = 0{\cdot}73$, the constant $b = 1{\cdot}23$; hence it follows that

$$P/P_0 = 0{\cdot}73\,(I/I_0)^{1{\cdot}23}. \qquad \ldots\ldots(2)$$

[1] The experiments have been carried out by A. Andreev, A. Bronstein and G. Gersuni.

By comparing equations (1) and (2) a quantitative estimate of the differences between the reaction of the cochlea to an alternating current as shown by (*a*) a normal auditory apparatus and (*b*) one showing subnormal hearing will be clearly seen. The differences revealed are so great that they lie beyond the limits of any possible errors.

Discussion

It is not easy to estimate the phenomena observed when alternating current is passed through the auditory apparatus. One thing, however, is clear: all the characteristic features of electrical stimulation, which have been previously described, are due to the effect produced by alternating current on certain structures within the cochlea, and are dependent on the condition of these latter [see Gersuni & Volokhov, 1937]. However, the alternative hypotheses as to whether the passage of alternating current evokes mechanical vibration or whether the effect produced by the current is due to its immediate action on the receptor elements can so far hardly be said to have been satisfactorily differentiated.[1] It may, however, be pointed out that if it be assumed that the passage of alternating current evokes vibration of the cochlear fluid similar to that occurring with adequate stimulation, it would be hard to account for the fact that intensities corresponding to sensation levels of 25–30 db. result in marked distortion of the perceived tone. Sound stimulation corresponding to a sensation level of 25–30 db. produces tones which are absolutely pure, while subjective harmonics are entirely inaudible.

On these grounds it may be supposed that our data suggest the other interpretation of the phenomena, viz. that the immediate effect of alternating current is on the receptor elements of the cochlea, without the electrical forces being necessarily transformed into mechanical ones.

Certain quantitative comparisons between the alternating potentials arising in the cochlea as a result of the action of sound and the effect of conveying alternating current to the cochlea from outside also seem to be in favour of the latter assumption.

The data reported by Davis *et al.* [1934] show the relation between the potentials generated within the cochlea (Wever and Bray pheno-

[1] So, for instance, the interaction between electrical and the sound stimulation which has been previously described (the production of beats by simultaneous electrical and sound stimulation at approximately similar frequencies [Gersuni & Volokhov, 1936]) may be dependent either on the interaction of two mechanical or of two electrical processes developing in the cochlea. This latter possibility was kindly suggested to us by Prof. A. V. Hill.

menon) and the sound pressure affecting the ear. These results are shown in Fig. 3, where the ratio between the logarithm of the sound pressure (P/P_0) and the magnitude of the logarithm of the resultant potentials (V/V_0) is expressed by the straight line (III).[1] We have plotted only those points lying not higher than 40 db. above threshold for all frequencies. As may be seen from this graph, the straight line (III) runs between the straight lines (I) and (II). This means that the relation between the sound pressure and the potentials generated within the cochlea lies within the same range of magnitude as the relation between the sound pressure corresponding to the given sensation level and the magnitude of an alternating current stimulus in electrical stimulation of the ear.

This coincidence of phenomena can be most readily explained if it be assumed that the alternating current conveyed from outside immediately affects those biological processes developing in the receptor elements of the cochlea which are likewise affected by alternating potentials arising in the cochlea itself. In this case a close relationship must exist between electrical stimulation and the mechanism of a natural stimulation. It must be stated, however, that this assumption must be regarded only as hypothetical.

A similar hypothesis has been suggested independently by Zotterman [1935], though apparently it was not based on any quantitative data, to judge by the very brief report which we have read.

In summarizing the foregoing data, we are thus led to conclude that the mechanism by which alternating current affects the cochlea is not so far sufficiently clear. And, though direct action of applied alternating current on the receptor elements of the cochlea appears to be highly probable, the possibility that mechanical vibration is generated therein can scarcely be rejected as yet.

Summary

1. The auditory sensations arising when alternating current is passed through a normal auditory apparatus differ in some respects from those produced by a sound stimulation of the same frequency.

2. The relation between the sensation level N [$(=20 \log P/P_0)$, where P and P_0 are sound pressures] and current strength I, necessary to produce a sensation of equal loudness, can be expressed by the equation: $P/P_0 = 1{\cdot}18\,(I/I_0)^{1{\cdot}3}$.

[1] Davis's original curve is drawn on a half logarithmic scale (see Fig. 8 in his paper).

3. The phenomena described as resulting from the action of alternating current on the inner ear may depend either on the production of mechanical vibration in certain portions of the cochlea, or on the immediate action of the current on the receptor elements of Corti's organ.

4. The importance of electrical stimulation as a method of studying the functions of the auditory apparatus is emphasized.

We express our gratitude to Prof. L. A. Orbeli for the constant interest he has shown in our work.

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IMPEDANCE CHANGES IN MUSCLE DURING CONTRACTION, AND THEIR POSSIBLE RELATION TO CHEMICAL PROCESSES

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Introduction

In several recent publications [Dubuisson, 1933, 1934*a*, *b*, *c*, *d*, 1935*a*, *b*] I have recorded the changes of electric impedance of muscle before and during contraction. The records showed: (*a*) a rapid decrease of impedance (the *a* wave) preceding the mechanical contraction, and (*b*) a second decrease of impedance (the *b* wave) accompanying the contraction itself. The *a* and *b* waves occur simultaneously with the *R* and *T* potentials of the electromyogram.

Since then, Bozler [1935] has studied the question. He refers to the first paper [Dubuisson, 1933] which dealt only with the impedance change (*b* wave) accompanying contraction. With a frog's gastrocnemius he obtained results similar to mine, but with a sartorius he found an increase, not a decrease, of impedance. The question, therefore, has been reinvestigated, particularly in view of a personal suggestion from Dr von Muralt, that a comparison should be made between the impedance changes and the chemical processes known to take place in muscle. After systematic repetition of Bozler's experiments and my own the reason of our disagreement has been found. Bozler's method allowed better isometric conditions to be attained. If the muscle does not shorten at all, there is a clear rise of impedance [cp. Achelis, 1932]: shortening of the fibres, however, is accompanied by a lowering of impedance. Under imperfect isometric conditions there is interference between these two effects. Thus the disagreement between the many investigators of this problem can be explained [MacClendon, 1927; Achelis, 1932; Buchthal, 1934–5; Buchthal & Péterfi, 1934]. When mechanical shortening is completely avoided, the impedance changes are of a character which suggests a relation to the chemical processes known to occur in muscle.

Method

The muscle is introduced into one branch of a Wheatstone bridge, its impedance being compensated by a resistance R, in series with a resistance r which is shunted by a capacity C. The impedance of this system contains an impedance $Z_0 = R + \frac{r}{1+C^2\omega^2r^2}$ and a reactance $Z_i = \frac{C\omega r^2}{1+C^2\omega^2r^2}$ ($\omega = 2\pi \times$ frequency).

By having a measured disequilibrium in the bridge before any excitation of the muscle, one can determine sign and extent of the impedance variation in the tissue. By varying the resistance (R or r) or the capacity (C), one can determine whether resistance and reactance changes have the same or a different sign.[1]

The Wheatstone bridge (Fig. 1) is connected to a low-frequency (2000 cycles per sec.) or a high-frequency (1,200,000 per sec.) oscillator. The latter is controlled by a piezo-electric quartz crystal, introduced into the grid circuit of the valve. With low-frequency determinations, the current leaving the bridge passes through a two-stage transformer-coupled amplifier. With high frequency, the current is heterodyned and transformed into oscillations of about 2000 cycles per sec. which are sent through the amplifier. For technical details see Fig. 1.

Five muscle preparations were used: sartorius, gastrocnemius, semimembranosus, rectus and obliquus abdominis, either from Belgian or from Hungarian frogs.

Three types of electrodes were employed:

(1) The muscle (gastrocnemius or sartorius), *in vivo* and *in situ*, was fixed and compressed transversely by two circular chlorided silver electrodes, partially covered with gum-lacquer [see Dubuisson, 1933, 1934*a*].

(2) The muscle (rectus or obliquus abdominis) is tied over the opening of a test-tube (Fig. 2). This tube is filled with phosphate Ringer[2] and immersed in a bath of Ringer. The electrodes are platinized platinum discs of 3 mm. diameter, one of them being inside the tube, the other one facing the muscle from the outside solution. The tube communicates on one side through a cock with a pipette which permits variation in the

[1] In some previous papers I represented the reactance variation by $d\frac{1}{C\omega}$ and the resistance changes by dr and dR. This was simplified, and actually inexact; it should be

$$d\frac{C\omega r^2}{1+C^2\omega^2r^2} \quad \text{and} \quad d\frac{r}{1+C^2\omega^2r^2}.$$

[2] NaCl, 0·65 g.; KCl, 0·014 g.; $CaCl_2$, 0·012 g.; $NaHCO_3$, 0·02 g.; NaH_2PO_4, 0·001 g.; Glucose, 0·2 g.; Aq. dest. ad 100 c.c.

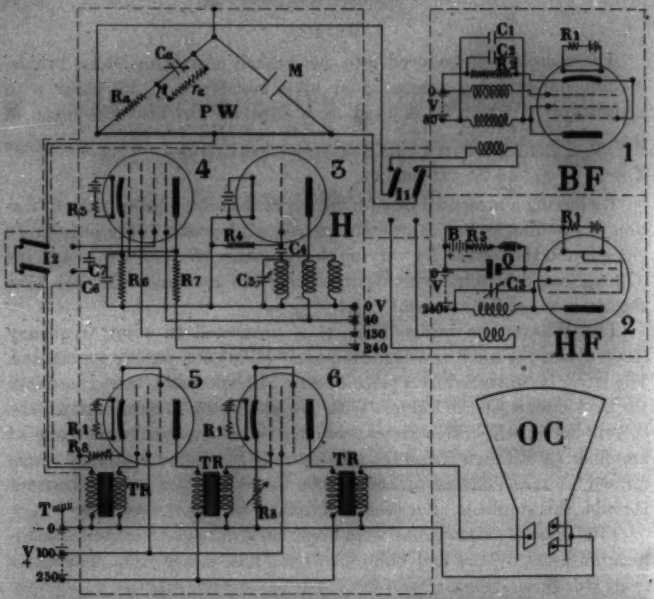

Fig. 1. Circuit diagram of impedance determination during muscle contraction. *Bridge: PW*, Wheatstone bridge containing the muscle *M*, the variable resistances r_c (0–15,000 ohms), R_8 (0–800 ohms) and the capacity C_8 (0–1 μF.); *i*, key closed during low-frequency determinations. *Oscillator:* The bridge is supplied either by a high-frequency current produced by the oscillator *HF*, or by a low-frequency oscillator *BF* by using the key I_1. *HF*, 1,200,000 cycles/sec.; *Q*, piezo-electric quartz crystal; 2, valve 47; R_1, 1·5 ohms; R_2, 50,000 ohms; C_2, 0·001 μF.; *B*, battery 16 V. *BF*, 2000 cycles/sec.; 1, valve 2 A5; R_1, 1·5 ohms; R_2, 500 ohms; C_1, 0·025 μF.; C_2, 2 μF. *Heterodyne, H:* Valve 3 (Philips, A 410) acts as oscillator, valve 4 (2 A7) as modulator. It modulates the oscillations generated by valve 3 with those from the bridge supplied with *HF*. The modulation frequency is regulated to 2000 cycles/sec. This heterodyne is only used for high-frequency determinations. A key I_2 allows it to be removed from circuit. R_4, 100,000 ohms; R_5, 1·87 ohms; R_6, 250 ohms; R_7, 150,000 ohms; C_4, 0·00025 μF.; C_5, 0·00005 μF.; C_6, 0·5 μF.; C_7, 0·02 μF. *Amplifier:* 5 and 6, valves 57; R_1, 1·5 ohms; R_2, 5000 ohms; *TR*, transformers. The broken lines indicate the shielding earthed in *T*; *OC*, cathode-ray oscillograph, the circuit of which is not shown.

pressure within the tube and distension of the muscle, fixed on the end of the tube. By closing the cock, one can maintain a constant distension and keep the muscle in isometric conditions. This is the same arrangement

as used by Achelis [1932] and Quensel [1932]. This procedure is very satisfactory, but unfortunately can be applied only to muscles with a large surface such as the rectus and obliquus abdominis. I tried to use tubes of different diameter, varying from 2 to 16 mm. The narrow tubes which might carry a sartorius of a large frog generally give preparations of reduced excitability (perhaps because the ligature damages a relatively greater part of the muscle). On the other hand, muscles tied over tubes of 16 mm. diameter cannot very easily be fixed. During stimulation only parts of the preparation respond, which produce undulating movements of some fibres and abolish isometric conditions. When using a

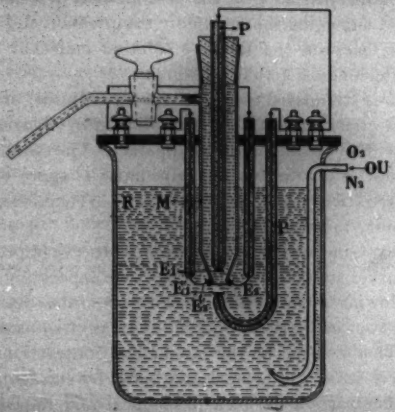

Fig. 2. Electrodes for the abdominal muscles: E_1 and E'_1, platinized Pt electrodes; E_2 and E'_2, stimulating electrodes applied to the muscle (see text); *R*, wall of a Becker glass; *M*, wall of test-tube; *P*, paraffin; *OU*, tube to permit bubbling with O_2 or N_2.

very strong stimulating current in order to get the whole muscle to contract simultaneously, the tissue becomes polarized and this affects the records considerably. I generally used tubes of 6–8 mm. diameter, which gave the most consistent results. The main advantage of using the test-tube method is in avoiding the mechanical damage following the compression by metal electrodes and in allowing the study of impedance changes during various chemical conditions. Before taking a reading one has to wait until the impedance of the resting muscle becomes stable. Generally the impedance diminishes for a certain time, due, probably, either to a gradual equilibration with the Ringer's solution, or to the after-

effects of the injury produced by dissection. After 30 min. the preparations are generally in a stable condition.

(3) In certain experiments a similar arrangement was used to that described by Bozler [1935] and Bozler & Cole [1935]. The muscle (sartorius, gastrocnemius, semimembranosus) is compressed between two ebonite plates which are perforated symmetrically by two platinized platinum wires of 0·2 mm. diameter. It is rigidly attached at both ends to one of these plates. The whole preparation dips into a bath of phosphate Ringer. The main advantage of this method is that one may use any kind of muscle and obtain excellent fixation; the isometric conditions are perfect. On the other hand, just as with the first method described, it has the disadvantage of damaging the muscle by compression and of preventing communication between the Ringer's solution and that region of the muscle with which one is actually dealing.

The muscle is stimulated indirectly (gastrocnemius) or directly with chlorided silver wires. I used a Neon lamp stimulator of variable frequency [Scheminzky, 1930] or an induction apparatus. The stimulating current may, sometimes, greatly affect the impedance changes of the muscle, producing polarization phenomena in the tissue. Therefore, preparations of low excitability must not be used.

In certain experiments it was of interest to observe impedance changes and tension curves simultaneously. These were separately recorded, the time marks allowing a comparison of the two records. Since the muscles were generally kept under isometric conditions as strict as possible, it was often very difficult to record the mechanical contraction. I succeeded only by using electrodes of types 1 and 3 and by enlarging the records. Further, the comparison between mechanical and impedance changes referred to in the text are only approximate (± 10 msec.). It will be shown below that such an error is insignificant except when dealing with the start of the contraction.

Results

Impedance changes in normal muscle

The sign of the changes. I repeated a whole series of experiments with the gastrocnemius, using the same arrangement as before (electrode 1, see above).

Under these conditions the high-frequency resistance of the muscle does not change appreciably during contraction.

With low frequency the results were in agreement with my previous observations: there is a lowering of impedance simultaneously with the

contraction which is independent of the form, size and distance of the electrodes. This phenomenon was so regular that one had to discard the hypothesis of an artefact, due to contact variations between electrodes and muscle during its activity. The difference between Bozler's and my results could only be due to the fact that, in my experiments, the muscle fibres shortened somewhat. This was quite likely, since I mostly used Hungarian frogs; these are usually large and their powerful muscles difficult to fix. I repeated my experiments also with gastrocnemius muscles of small Belgian frogs, the weight of which did not exceed 25 g. I obtained a $-b$ wave when knee and foot were simply fixed on a supporting board, the foot in the direction of the leg. If, however, the foot is bent against the leg so that the gastrocnemius is in hyperextension, the impedance rises during the contraction. By modifying the position of the foot in respect of the limb one may pass from one variation to the other. In order to understand these facts one has to assume that the hyperextension produces better isometric conditions than the simple extension. This holds well for all the muscles examined and all kinds of electrodes used. As will be shown below, the positive change has not the same time characteristic as the negative, so that these two opposite impedance changes never cancel each other completely.

Records from normal muscles in non-isometric condition

The tracings show a lowering of impedance which is the greater the more the fibres are allowed to shorten. It has been described in detail in previous publications [see Dubuisson, 1935*c*]. I called it the b wave. Its start, maximum and end correspond to the start, maximum and end of the contraction. This $-b$ wave, therefore, could hardly bring further information about the mechanism of the contraction than does the corresponding mechanical tracing.

There are serious difficulties in avoiding completely a shortening of the fibres. Even if the length of the whole fibre does not vary, one part of it may shorten while another part is lengthened. One needs only to observe microscopically several dissociated fibres of a sartorius, the ends of which are rigidly fixed. During the first moments of a tetanic contraction, the whole of the fibres seems to be affected homogeneously; later, however, the contraction is often maintained more intensely in certain regions; there is a shortening in some parts, a lengthening in the others. During tetanic contraction it was also most difficult to avoid the lowering of impedance which accompanies the shortening of the fibres.

Changes during strictly isometric conditions

Single twitch. Under these conditions there is a rise of impedance during the contraction when measuring with low frequency. (With high frequency there is no appreciable change.) This phenomenon has been found by Achelis [1932] with tetanic stimulation of the obliquus abdominis and by Bozler [1935] on the sartorius, during single twitches and tetanic contractions. Bozler's records show that, during the single

Fig. 3. 1 and 2, Exp. B 76, gastrocnemius. In 1, nine twitches, muscle fresh and oxygenated; in 2, seven twitches of same muscle after seventy-five previous stimulations ($+b_1$ and $+b_2$ are now well distinct). Maximal deflexion 4 p.c.; temp. 7° C.; electrodes 3; indirect excitation. 3, Exp. B 62, obliquus abdominis. Twitch followed by contracture. Maximal deviation 18 p.c.; temp. 21° C.; electrodes 2; direct excitation. 4, Exp. B 71 *b*, sartorius, single twitches. Maximum deviation 7 p.c.; temp. 19° C.; electrodes 1; direct excitation.

twitch, the maximum impedance occurs after the isometric tension has reached its maximum, and that the impedance has not returned to its initial level when the mechanical contraction is already finished. This statement might suggest that the impedance changes have some relation to chemical processes. Bozler, however, comes to a negative conclusion.

During a single twitch, obtained by direct or indirect stimulation of a fresh oxygenated muscle, the form of the impedance change depends upon the kind of muscle used. In the sartorius and semimembranosus, the increase in impedance rapidly reaches a maximum, then falls quickly to one-eighth of its maximum value and returns slowly to its initial level. The maximum change amounts to not more than 10 p.c. of the resistance r.

The capacity C increases at the same time. In the gastrocnemius there is also a quick rise and a peak, while the disappearance, at first rapid, becomes slower already at one-quarter of the maximum height. In the rectus and obliquus abdominis the slowing of the rate of disappearance becomes distinct even at one-half of the maximum height. Apart from these differences, significant for the different kinds of muscles, there are also some individual variations. Three successive phenomena, however, are found without exception: quick rise, quick fall, and then slower disappearance.

When using a high recording speed, one finds that the rise of impedance actually consists of two partly asynchronous changes, since one often observes not one but two peaks: the first being the maximum of a rapidly rising and falling curve, the second belonging to another curve the rising part of which, although masked by the first wave, appears to be rather

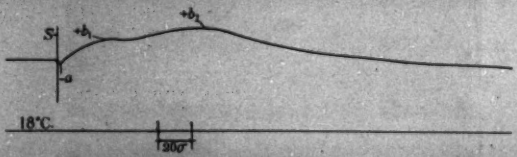

Fig. 4. Exp. B 7, rectus abdominis oxygenated and fresh. Isolated twitch recorded at high speed. At S, stimulus, one sees the waves $-a$, $+b_1$ and $+b_2$. Maximum deviation 2 p.c.; temp. 18° C.; electrodes 2; direct excitation.

slow, and the disappearance of which is much slower than that of the first wave. To facilitate discussion, I shall call these two impedance changes $+b_1$ and $+b_2$.

In fresh preparations the two waves are usually not discrete, except in the abdominal muscle (Fig. 4). Under certain conditions, however, particularly during fatigue, the double wave may become marked in any muscle. With increased fatigue the rapid initial wave ($+b_1$) becomes smaller; the second wave ($+b_2$), however, slower and spread out (Figs. 3 and 5). At a certain stage of fatigue both processes are of equal amplitude. Records obtained with high paper speed show usually a clear double peak or, sometimes, a flattened maximum in which it is difficult to distinguish the two waves. With further prolonged stimulation the changes become smaller and finally imperceptible, when the muscle is completely exhausted.

The tension curves, recorded simultaneously with the impedance changes, show that the point of maximum tension falls between the

maxima of $+b_1$ and $+b_2$. In other words, the first impedance rise reaches its maximum during the phase of rising tension; the second wave starts during the contraction and attains its maximum during the beginning of relaxation (fresh muscle) or at its end (fatigued muscle). At 18° C. the peak of the tension is at about 90 msec. after the start of the contraction (± 10 msec. error); the peak of $+b_1$ at about 30–50 msec., the peak of $+b_2$ (fresh muscle) at about 90–130 msec. In a fatigued muscle the peak of $+b_2$ lies near 180–250 msec. The high speed records do not indicate

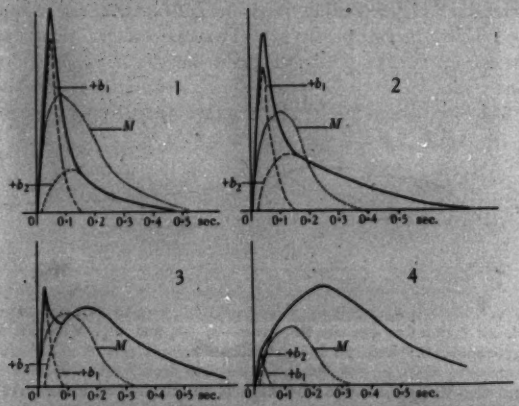

Fig. 5. Exp. A 104, oxygenated sartorius. Schema of four records during fatigue. Full lines: impedance changes; dotted *M*, myogram, taken separately and drawn at the same scale. In $+b_1$ and $+b_2$ the supposed curve of the two impedance changes; temp. 18° C.; electrodes 3; direct excitation.

a perfect synchronism between the beginning of the contraction and $+b_1$. Although the end of the $-a$ wave (preceding contraction) cannot be clearly distinguished from the beginning of the $+b_1$ wave, since its start is not sufficiently steep, the actual contraction seemed to start always a few milliseconds after the beginning of the $+b_1$ wave. The start of $+b_2$, masked by $+b_1$, is difficult to determine: it lies within the first 25 msec. following the beginning of the contraction.

In all cases the contraction disappears before the impedance has returned to the initial value; the remainder of the impedance change disappears in one or several seconds.

In the abdominal muscles one often finds that the single twitch consists of a rapid contraction followed by a much slower contracture which starts before complete relaxation of the initial contraction. The corresponding impedance changes show a much larger $+b_2$ at a greater distance from $+b_1$ than is usual (Fig. 3 (3)). There are all possible variations

Fig. 6. 1, Exp. A 36, obliquus abdominis. First of a series of tetani of 4 sec. duration in fresh well-oxygenated muscle. Maximum deviation 15 p.c. of r; temp. 14° C. 2, Exp. B 73, rectus abdominis. Seven successive tetani of 2 sec. Oxygenated muscle. Maximum deviation 11 p.c.; temp. 19° C.; electrodes 2; direct excitation. 3, Exp. B 86*c*, sartorius. Five successive 2 sec. tetani. Oxygenated muscle. Maximum deviation 19 p.c.; temp. 18° C.; electrodes 3; direct excitation.

between a twitch with or without obvious contracture. Closer examination gives the impression that there are only quantitative differences between the impedance changes during a normal twitch and those during twitch with contracture, the latter merely showing a lengthening and a much greater amplitude of the second impedance change. The mechanical records give the same impression: the relaxation of a single twitch always has a more or less delayed appearance, particularly during fatigue.[1]

[1] The majority of records published elsewhere [see e.g. Neumann, 1932; and Kraus *et al.* 1932] show this phenomenon.

Tetanus. Repetitive excitation at a sufficient frequency (e.g. once in 5 sec.) produces a summation of the remainders of impedance change (Fig. 3 (4)). The rate of this summation increases with frequency of stimulation.

During a complete tetanus (Fig. 6) the impedance rises immediately after the onset of stimulation, at first rapidly, then more slowly during the whole period of excitation (increase of r and of C). After removing the stimulus the impedance falls, at first very quickly, then more slowly and reaches its initial level only after several minutes. The size of the initial impedance rise varies from preparation to preparation. The higher the initial rise, the larger is the rapid fall immediately following the end of stimulation. When stimulating a muscle slowly at first, then with increasing frequency, one can show that this quick initial rise and rapid fall of the tetanus curve corresponds to the $+b_1$ waves of the single twitches; the slow progressive impedance change during the tetanus, however, has to be attributed to the partly summing $+b_2$ waves. In those muscles which show a large $+b_1$, as compared with $+b_2$, the rapid changes during tetanic contraction are also relatively larger (sartorius).

The impedance change produced in a fresh and well-oxygenated muscle by a 2 sec. tetanus does not return to the initial level before 3 or 4 min. (temperature 18° C.). Comparing, however, the impedance changes during successive 2 sec. tetani, separated by resting periods of 4–5 sec., one finds that the initial drop after cessation of stimulation is slower after the first than after the succeeding tetani: the remainder disappears more rapidly in a slightly fatigued muscle than in an entirely fresh preparation (Fig. 6 (2)).[1]

Anaerobic conditions, strictly isometric contraction

Electrodes of type 2 are most suitable for studying the impedance changes in anaerobic conditions. The test-tube carrying the muscle is filled with O_2-free Ringer and immersed in a solute saturated with N_2 (the nitrogen being completely deprived of oxygen by passing through an alkaline sodium hydrosulphite solution). Very consistent results are also obtained by poisoning the muscle with KCN (M/1000). The diffusion of KCN into the tissue is very quick; after a few minutes the respiration has completely stopped.

During anaerobic contraction (twitch or tetanus) there is always a remainder of impedance rise, the more distinct the more often the

[1] See also p. 144.

stimulation has been repeated. The other properties of the $+b_1$ and $+b_2$ waves, however, are the same in aerobic and anaerobic conditions.

Fig. 7 shows the result of impedance determinations on the abdominal muscle, using electrode 2. In A, two successive tetani of 5 sec. duration

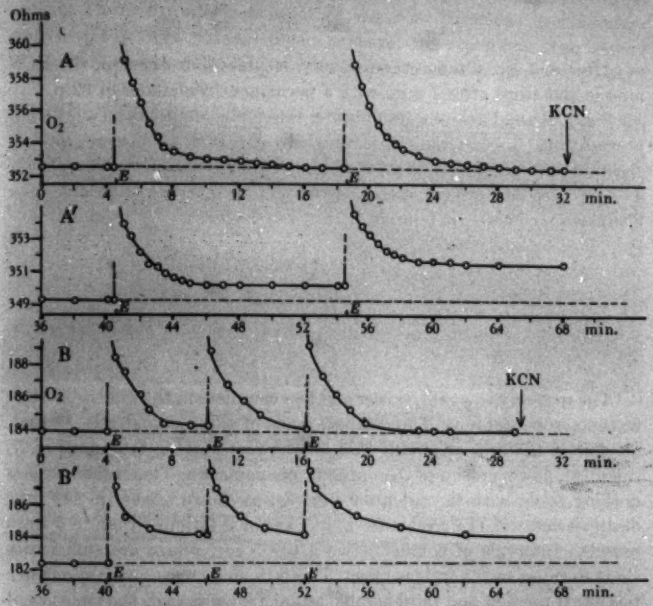

Fig. 7. A and A′, Exp. B 55, rectus abdominis, in Ringer for 45 min. In A, oxygenated muscle: *E*, 5 sec. tetanus; impedance changes (*r* and *C*) are followed by telephone and noted about each min.; changes of *C* and *r* are parallel and of the same sense; one sees that after a 5 sec. tetanus the impedance does not return to the initial level before about 10 min.; after 32 min., 42 mg. KCN added to 300 c.c. Ringer. In A′, continuation of A, anaerobic; in *E*, 5 sec. tetanus; temp. 19·5° C.; electrodes 2; direct excitation. B and B′, Exp. B 50, like A; in B, aerobic; in B′, anaerobic; temp. 21° C.; electrodes 2; direct excitation (see text).

and 14 min. interval are shown; in A′, the changes in the same muscle, after KCN poisoning. The first anaerobic tetanus started 22 min. after the last aerobic tetanus. The comparison of the progressive disappearance

of the remainders after the first aerobic and anaerobic tetanus is tabulated (in p.c.) below:

Time in min.	Aerobic	Anaerobic
1	22	18
2	58	48
3	79	65
4	85	78
14	100	78

After a 5 sec. tetanus the recovery is somewhat slower in the KCN muscle and stops after 4 min. with a permanent remainder of 22 p.c.

Fig. 7, B and B′ show a result under the same conditions on a different preparation. 5 sec. tetani with intervals of 6 min. only were applied. B shows three aerobic, B′ three anaerobic tetani. Between the last aerobic and the first anaerobic stimulation there is an interval of 24 min. The comparison of the first tetanus of each series gives:

Time in min.	Aerobic	Anaerobic
1	15	22
2	46	47
3	73	57
4	87	61
6	88	62

The recovery is, again, slower and less complete in the anaerobic than in the aerobic tetanus. The disappearance of the remainder is, at first, quicker in the first anaerobic than in the first aerobic tetanus; Fig. 7, A and A′, however, shows the opposite phenomenon. This difference has nothing to do with the oxidation process: as shown above (p. 142), the disappearance of the remainder is quicker in a fatigued than in a fresh muscle. Intervals of 6 min. between the 5 sec. tetani are sufficiently short to cause such a fatigue effect. This fatigue increases with successive tetani. If the first and third aerobic tetani are compared, the recovery of the impedance in each case follows the time course shown below:

Time in min.	First tetanus	Third tetanus
1	15	36
2	46	57
3	73	77
4	87	92
6	88	100

Monoiodo-acetate poisoning, strictly isometric conditions

Abdominal muscles with electrode 2, and sartorius, semimembranosus and gastrocnemius with electrode 3 were used.

In the first experiments the muscles were kept for 50 min. (18–20° C.) in oxygenated phosphate Ringer, to which monoiodo- or monobromo-acetate (1/20,000) was added. The bath was renewed twice in 25 minutes' interval. The muscle was then rigidly fixed, the electrodes adjusted, and the whole preparation immersed in phosphate Ringer free from oxygen. The first results were negative, longer intoxication times therefore were used (up to 1½ hours).[1] Then, preparations which had been soaked in monoiodo-acetate for at least 1 hr. 5 min., and still gave perfectly

Fig. 8. 1, Exp. B 63, obliquus abdominis, poisoned for 1 hr. 10 min. by oxygenated phosphate Ringer, to which monobromo-acetate (1/20,000) had been added; temp. 18° C.; seven tetani of 4 sec. duration. The permanent remainder corresponds finally to an increase of impedance of 17 p.c.; direct excitation. 2, Exp. B 74, sartorius poisoned for 70 min. at 10° C.; eight twitches; electrode 3; direct excitation. 3 and 4, Exp. B 89, semimembranosus poisoned for 1 hr. 5 min. at 22° C.; single twitches; electrodes 3; direct excitation.

normal contractions, showed a peculiar impedance change. Each contraction is followed by a distinct persistent remainder. Comparison with normal records shows that this remainder actually consists of the $+b_2$ wave. This wave, in the poisoned muscle, maintains its maximum level. This irreversible persistence of $+b_2$ follows even the first contraction, thus long before rigor occurs.

The $+b_1$ wave seems hardly to be affected by the poisoning. This, however, needs further study with high recording speed. Hitherto, the

[1] There are no safe methods yet of poisoning a muscle just sufficiently to stop the lactic acid formation. Generally one has to take account of seasonal and temperature influences (there is a considerable effect of temperature on the time required for intoxication, see Ghaffar, 1935). It is further known that the actual intoxication occurs only in rather narrow limits of time.

only possible alteration of $+b_1$ which might be perceptible in a small number of records is a slight reduction of its total duration.

Tetani of poisoned muscles show corresponding results: the impedance develops normally during stimulation, falls only slightly after cessation of the stimulus, and remains permanently at a high level. The partial recovery which immediately follows the end of tetanization is proportional to the height of the initial steep rise which follows the onset of stimulation. This corresponds to the normal tetanic records. The only difference is, therefore, the absence of the gradual disappearance of the impedance remainder in poisoned muscles. This is in agreement with the observations on the single twitch, showing a normal $+b_1$ and a persistent maximum of $+b_2$.

After four to six tetani of 2 sec. duration the muscle gets in rigor. The impedance stays irreversibly at a level distinctly above the normal values (this has been shown also by Quensel, 1932, and Bozler & Cole, 1935).

Discussion and conclusions

No conclusive evidence is available concerning the character of the first wave ($+b_1$). If $+b_1$ (and the other accompanying phenomena) represents a chemical process, then it must be a rather fast transformation hardly affected by monoiodo-acetate. A chemical substance of such properties actually described in the literature is adenyl pyrophosphoric acid [Lohmann, 1936], the breakdown of which is a quick exothermic reaction occurring before the hydrolysis of phosphagen (since adenylic acid formed during the breakdown of adenyl pyrophosphoric acid is the hydrolysing co-enzyme of the creatinephosphoric acid) and the synthesis of which is as rapid in a normal as in a monoiodo-acetate poisoned muscle.

If this similarity is not only chance the transformations of adenyl pyrophosphoric acid might perhaps be related directly or indirectly with the $+b_1$ wave and the accompanying physical phenomena.

In any case it seems likely that the $+b_1$ wave is connected with the fundamental process of the contraction since its relative amplitude seems to be proportional to the mechanical energy which the muscle can liberate. Why this physical phenomenon of a fundamental process is finished before the mechanical energy attains its maximum remains to be explained.

More definite evidence exists about the second wave ($+b_2$). The persistence of a remainder (22 p.c.) in anaerobic conditions and the absence of recovery after poisoning with monoiodo-acetate indicate that the $+b_2$

wave is related somehow to the phosphagen complex. Thus, phosphagen would break down during relaxation. On the other hand the experiments lead to the conclusion that the $+b_2$ wave—although coming in during the relaxation—is a factor of contraction, since it is larger when the relaxation is slow or when the contraction is followed by an obvious contracture. The $+b_2$ wave would correspond therefore to some kind of a delayed contraction which would be present in any twitch and must be distinguished from the initial fundamental process.

It is impossible to make more than hypothetical assumptions about the actual mechanism of the impedance rise, directly or indirectly related to chemical transformations. The rises of impedance $+b_1$ and $+b_2$ result from simultaneous increases in the ohmic resistance and in the capacity (r and C). We do not know, however, whether these changes are the result of changes in the molecular structure or of displacements of certain dielectrics like water [see d'Ancona, 1932]. According to von Muralt, the reduction of double refraction during the contraction must be attributed to myosine and it is not impossible also that the impedance changes might be attributed to such structures, since changes in the optical qualities of myosine must correspond to changes in the dielectric constant [von Muralt, 1935]. It is, however, uncertain whether the two impedance changes—just as the two changes in double refraction—are localized in the same structure.

The impedance changes during contraction are analogous to other physical phenomena of the contraction.

1. According to the works of Hill & Hartree, the initial heat production of a single twitch has two phases: the first heat liberation occurs when the tension starts rising, the second during relaxation. These two heat productions coincide with the two impedance changes. The analogy between these two different phenomena may be followed in detail.

A suitable way for the comparison of these different observations is to use not absolute time units, but the interval between stimulation and point of maximum tension as relative unit, thus making comparable the observations on different kinds of frogs (*R. temporaria* [Hartree] and *R. esculenta* [Dubuisson]) at different temperatures and seasons. One then finds a very good agreement (Fig. 9). The analogy still holds for fatigued muscles. The first heat production, just as the height of $+b_1$, is smaller than the normal; the second heat production is not diminished, but its maximum is delayed (at 0° C. up to 700 msec. from the moment of excitation); $+b_2$ also is not diminished, but is often even larger and always shows a delayed maximum (at 0° C. at 750 msec. after excitation).

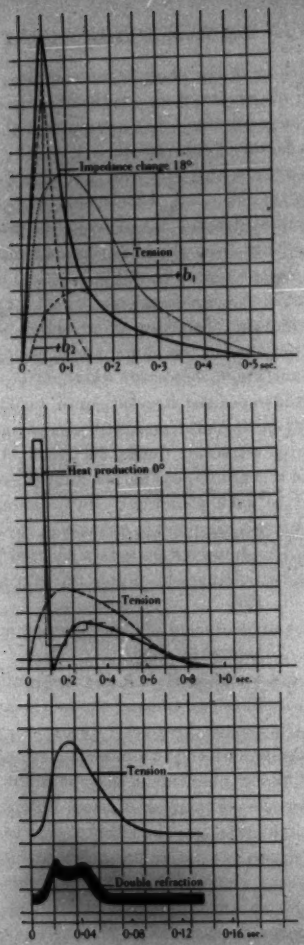

Fig. 9. Above: impedance changes during isometric contraction of fresh oxygenated sartorius; temp. 18° C. Middle: initial heat production (from Hartree, 1933, Fig. 1*a*); fresh sartorius; temp. 0° C. Below: diminution of double refraction (after von Muralt, 1932, Fig. 3); fresh sartorius. The three figures are adjusted so that the maximum tension in the three cases coincides. Abscissæ: time. The analogy between $+b_1$, first initial heat, first diminution of double refraction (all three during the period of rising tension), is apparent, as is also the analogy between $+b_2$, second initial heat and second diminution of double refraction (all three during relaxation).

2. von Muralt [1932], studying the changes in double refraction of the anisotropic regions of the sartorius during isometric contraction, found two successive changes: the first occurring just in the beginning of the contraction, the second developing during the relaxation. He further observed that the second change becomes larger and lengthened during fatigue. This also is similar to the impedance changes. The correspondence in time between von Muralt's records and my own seems to be not very striking at first sight, but, when using as measure not absolute time units, but the interval between stimulus and maximum tension, the analogy becomes perfect (Fig. 9).

3. The same striking analogy is found for tetanic contraction. Hill and Hartree [1920] have shown that the tension during a tetanus can be maintained only by a continuous liberation of energy, which appears as a continuous production of initial heat. The impedance changes equally show a process which increases during the whole period of stimulation, confirming Bozler's observations.

4. The tetanic impedance changes coincide also with the volume contraction of the muscle. Discovered by Ernst [1925], it has been studied in detail by Meyerhof & Möhle [1933, 1936], Meyerhof & Hartmann [1934] and Hartmann [1934]. Immediately after the beginning of the tetanic contraction the muscle volume diminishes, at first rapidly, then more smoothly. After cessation of the stimulus the volume increases, at first rapidly, then more slowly.

5. E. von Baeyer & A. von Muralt [1934] further observed that the translucency of muscle changes during fatigue, and von Muralt [1934] studied the translucency changes during contraction. He obtained curves closely analogous to the impedance changes during tetanus (cf. Fig. 7 with the figure 4 of von Muralt [1934]).

Heat production, double refraction, volume contraction, translucency changes and impedance changes show such similar time relations that one may assume that these different physical processes are closely related to each other. This is suggested, still more convincingly, when comparing the results with anaerobic or poisoned muscles (monohalogenoacetate) obtained with these different methods as shown below:

6. The work of Meyerhof, Möhle and Hartmann on the volume changes during anaerobic contraction has shown that there is a persistent remainder after a tetanus. This remainder corresponds to a certain amount of decomposed creatinephosphoric acid which, under anaerobic conditions, cannot be resynthesized. The volume increase following the cessation of a tetanus would represent only the anaerobic synthesis of phosphagen.

Conversely the volume contraction accompanying the tetanus would be due to the breakdown of creatinephosphoric acid (and also of adenylphosphoric acid). In an O_2-free environment, von Muralt observed that the translucency of muscle does not return to the normal value after a tetanus. In these records the relatively rapid recovery of translucency would be a consequence of the anaerobic synthesis, the remainder would correspond to a certain amount of decomposed phosphagen.

Poisoning with monohalogeno-acetate, according to Lundsgaard [1930], prevents the transformation of mono- and di-hexosephosphoric ester into lactic acid; the anaerobic synthesis of phosphagen, however, can be effected in normal conditions only by the exothermic liberation of lactic acid. In a completely poisoned muscle, therefore, this synthesis cannot occur. Hartmann found, under these conditions, a considerable remainder of volume contraction increasing during successive tetanic contractions until contracture appeared. Von Muralt similarly observed a progressive loss of translucency.

All these results agree very well with the impedance changes, which show a remainder of 22 p.c. in anaerobic conditions and a much more significant remainder in the intoxicated muscles.

The analogy between these quite different physical observations is remarkable, whether considering a single twitch, or a tetanus, aerobic or anaerobic contraction and monoiodo-acetate poisoning.

Summary

1. In conditions which are not strictly isometric, contraction of a muscle is accompanied by a lowering of impedance ($-b$ wave), which is proportional to the shortening of the fibres.

2. In strictly isometric conditions the contraction is accompanied by a double successive rise in impedance. The first change ($+b_1$) starts probably several milliseconds before the contraction and attains its maximum during the phase of rising tension. The second ($+b_2$) reaches its maximum during the relaxation and returns with a distinct delay.

3. As the muscle fatigues the $+b_1$ wave becomes smaller, while $+b_2$ is lengthened and often becomes larger.

4. When the muscle contraction is followed by a more or less sustained contracture, the $+b_2$ wave is considerably larger than usual.

5. The two impedance rises $+b_1$ and $+b_2$ are analogous to the two diminutions in double refraction [von Muralt] and the two phases of initial heat production (Hill & Hartree). These different phenomena probably arise from the same causes.

6. During a tetanus there is at first a rapid rise of impedance, then, during the whole period of stimulation, a gradual increase. After the cessation of tetanization the impedance returns to its initial value at first rapidly then more slowly.

7. The tetanic impedance changes are homologous to the records of volume changes of muscle obtained under the same conditions of stimulation [Meyerhof, Möhle, Hartmann].

8. The rapid phases of rise and fall of impedance during and after tetanus correspond to the $+b_1$ waves of the single twitches; the slowly rising and falling portions to the $+b_2$ waves.

9. During anaerobic conditions there is a permanent remainder of impedance (22 p.c. of the maximum increase), as there is a remainder of volume contraction.

10. When the muscle is poisoned by monoiodo-acetate the $+b_2$ wave persists at its maximum (permanent remainder = 100 p.c.). Under these conditions, therefore, the impedance changes are analogous to the volume changes.

11. The following conclusions are drawn:

(*a*) $+b_1$ is perhaps related, directly or indirectly, to the transformations of adenyl-pyrophosphoric acid.

(*b*) $+b_1$ is related to the fundamental initial process of the contraction.

(*c*) $+b_2$ is related, directly or indirectly, to the transformations of phosphagen.

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THE EFFECTS UPON THE HUMAN ELECTROCARDIOGRAM OF THE INTRODUCTION OF CALCIUM AND POTASSIUM INTO THE BLOOD

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The purpose of this investigation is to ascertain the effects produced on the electrocardiogram by alterations in the concentration of the bases normally present in the blood. Numerous observations have been made on isolated and intact hearts of amphibians and lower mammals, but so far as the human subject is concerned only a few investigations are available.

Methods

The method employed consists in administering various bases to individuals, and taking electrocardiograms and blood samples before and after such administration. With the exception of those cases in which the calcium preparations were administered orally the bases were injected intravenously. After a preliminary blood sample had been taken from the median basilic vein, the patient was allowed to rest for about fifteen minutes. An electrocardiogram was next taken, after which the intravenous injection of the base was made into the basilic vein. The time occupied by the injection never exceeded two minutes. Additional electrocardiograms and blood samples were taken at varying times after the injection.

Calcium. The preparations used for intravenous injections were: Crookes's collosol calcium, Crookes's 10 p.c. solution of calcium lævulinate, and Parke-Davis's 10 p.c. solution of calcium chloride. For oral administration sodium-calcium-lactate together with parathyroid gland extract and also calcium gluconate with vitamin D, were used.

The quantities of calcium in the blood serum were determined by the technique described by Clark & Collip [1925], as an improvement on the method of Kramer & Tisdall [1921].

Potassium. Intravenous injections of various quantities of a 5 p.c. solution of potassium chloride (B.D.H.) were used in all these experiments.

The quantities of potassium in the blood serum were determined by the method of Kramer & Tisdall [1921].

We did not succeed in raising to any pronounced extent the serum sodium by injection of sodium chloride.

A rise in blood magnesium from 2·8 to 5·2 mg. per 100 c.c. did not affect the electrocardiogram in any particular way.

The tension of the string galvanometer was so adjusted that the passing through it of a current of 3 mV. would produce a deflection of 3 cm.

Results and discussion

Changes noted after the introduction of calcium into the blood stream (Fig. 1 and Table I)

Calcium salts were administered to six normal subjects. Fig. 1 and Table I indicate the typical changes observed. In the first place the cardiac cycle is markedly prolonged. This is mainly due to the lengthened period of diastole, i.e. *T-P*, but the duration of active contraction, *P-T*, also shows some prolongation, which averages 0·04 sec. and never exceeds 0·08 sec. The increased duration of *T-P* varies from 0·08 to 0·28 sec., but does not bear any relationship to the amount of increase in the serum calcium for the longest intervals are often observed with small increases of calcium and 30 and 60 min. after the injection when the calcium concentration has dropped considerably, and, in some instances, has even returned to its pre-injection value.

This applies in fact to all the changes observed in the electrocardiograms after calcium administration. It is remarkable that slight increases in the serum calcium should give rise to such pronounced effects, considering that they are for the most part within the normal variations of the calcium content of the serum.

At the moment we will not attempt to explain the matter. It is true the total calcium concentration in the blood has been determined, but we have not ascertained the ratio between free and combined calcium. We do not know exactly how calcium acts or how the diffusion of calcium from the serum after injection takes place; neither do we know whether the rate of diffusion from tissues such as spinal fluid is the same as that from the blood stream; and of course we do not know whether a slight increase in a particular base is sufficient to disturb the general equilibrium between all the bases.

The other changes which have been seen after the introduction of calcium, whether through oral administration or intravenous injection, are as follows:

The interval *Q-T* is generally prolonged, except in two cases where no change occurs. This prolongation amounts to 0·02–0·04 sec., and is evident at 10 min. after the injections and persists at 60 min. after the injections. The changes in the duration of the *T* wave are too inconstant to draw definite conclusions.

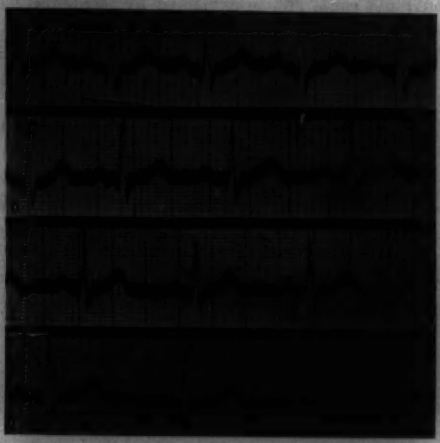

Fig. 1. J. W. 19-year male. Electrocardiographs (lead II) taken (A) before, (B) 10 min. after, (C) 30 min. after, and (D) 60 min. after intravenous injection of 2 c.c. collosol calcium. Time marker in twenty-fifths of a second.

TABLE I. J. W. 19-year male. Intravenous injection of 2 c.c. collosol calcium

Serum Ca concentration mg. per 100 c.c....	Before 10·2	10 min. after 10·4	30 min. after 10·4	60 min. after 10·2
	Durations (lead II), sec.			
P	0·12	0·10	0·10	0·10
P-R	0·16	0·18	0·16	0·16
Q-S	0·08	0·08	0·08	0·08
Q-T	0·28	0·32	0·34	0·32
T	0·16	0·20	0·20	0·20
P-T	0·44	0·48	0·48	0·48
T-P	0·08	0·14	0·16	0·20
	Heights (lead II), mm.			
P	3	2	2	1
R	17	15	14	14
T	4	4	4	4

The *P-R* interval is frequently prolonged by 0·02 sec.

The heights of the *P*, *R* and *T* deflexions are also changed. In general, the height of *P* is reduced, or is unchanged. The height of *R* too is frequently reduced.

Instances where the height of *P* drops from 7 to 5 mm., from 5 to 4 mm., and from 4 to 3 mm. frequently occur. There can be no doubt that all the changes in the electrocardiogram are due to the injection of calcium, because similar changes were observed in a considerable number of experiments and could not be explained by accidental variations, or enforced rest.

The decreased frequency of the heart beat has been observed by Lauter & Baumann [1927] in human subjects, and by Edwards & Page [1926] in dogs.

Kenji Fujimori [1933–4] administered 30 c.c. of a 3 p.c. solution of calcium chloride intravenously to human subjects and found that the duration of *P-P* and *P-Q* was prolonged, and that the heights of *P*, *R* and *T* were decreased. In these respects our findings agree with his observations. But he also stresses that the *Q-T* interval was shortened, which we did not find. This difference may be due to the fact that he based his findings chiefly on observations taken immediately and 3 min. after the injection, a period which we did not study.

It is known that stimulation of the vagus nerve produces an inhibition of cardiac action. Saito [1933–4] stimulated the vagus nerves of rabbits and noted the following changes on the electrocardiogram. A prolongation of the duration of *P-P* and *P-Q* and *Q-T* and a decrease in the heights of *P* and *T*.

The results obtained by vagus stimulation are so similar to the changes observed in our experiments with calcium administration in human subjects that one may be led to think that calcium acts on vagal nerve endings.

By means of an intramuscular injection of 1/50 gr. atropine sulphate it was possible to abolish the vagal influence in normal subjects with the following results: In two out of four cases the *P-R* interval is shortened, in the other two it remains unchanged. In all the duration of *Q-T* is reduced by 0·04 sec. In three the duration of *T* is diminished by 0·04 sec., and in all the *P-T* interval is shortened by 0·08 sec. The diastolic interval *T-P* is markedly shortened.

Having thus determined the effects produced by removing the vagus influence, the four cases were given on the following day an intravenous injection of calcium, together with 1/50 gr. of atropine. The following

changes were now observed: In one case in which the *P-R* interval was shortened by the atropine injection, it now retains its pre-injection value; in the other case it still remains shortened. In three cases the *Q-T* interval no longer shows a decrease, whereas in the fourth case it is actually increased by 0·04 sec. The same features are noticeable in the duration of *P-T*, and in respect of *T-P* the shortening is now much less marked than it was after the atropine injection alone.

From these results it would appear, therefore, that in spite of the vagus influence having been rendered ineffective, calcium has still a definite action on the heart.

Calcium and strophanthin. It is a well-known fact that strophanthin is not very effective for reducing the pulse rate in cases of hyperthyroidism. Therefore it would be of interest to note the action of calcium on this condition. The immediate problem was to ascertain whether the reduction of the pulse rate in normal subjects could be made more effective by adding calcium to strophanthin.

We were able to use four normal subjects to whom we administered on one day an intravenous injection of 1 mg. strophanthin, two days later an intravenous injection of a calcium product, and on the fifth day an intravenous injection of the same calcium solution, together with 1 mg. strophanthin. The changes noticed on the electrocardiograms taken before, 10 min. after and 60 min. after each injection can be described as follows:

After strophanthin only. In none of the cases are any changes recorded in the time durations of *P*, *P-R*, *Q-T* or *T*. The only change seen is that the diastolic interval *T-P* is prolonged: from 0·12 to 0·20 sec., from 0·08 to 0·16 sec., from 0·12 to 0·24 sec. and from 0·16 to 0·24 sec. in the cases respectively. This lengthening of *T-P* appears within 10 min. of the injection.

After calcium only. The changes are identical with those already noted after the introduction of calcium. The *T-P* intervals of the same four cases respectively show a lengthening which appears within 10 min. of the injection: From 0·24 to 0·40 sec., from 0·20 to 0·28 sec., from 0·16 to 0·34 sec. and from 0·20 to 0·36 sec. Within 60 min. the *T-P* interval tends to return to its pre-injection value.

After calcium plus strophanthin. Here the definite effects of calcium are evident in the decrease in duration of *P*, and of *Q-T* and *P-T*. The *T-P* intervals are prolonged in the four cases respectively as follows: From 0·20 to 0·36 sec., from 0·12 to 0·24 sec., from 0·16 to 0·32 sec. and from 0·20 to 0·40 sec. After 60 min. there is no appreciable change.

Now if we examine the changes in the time interval *T-P*; after

strophanthin only there is an average increase of 0·09 sec., after calcium only there is an average increase of 0·14 sec., whilst after calcium plus strophanthin there appears an average increase of 0·16 sec. Moreover, after calcium only the average increase of 0·14 sec. falls to 0·04 sec. within 60 min. of the injection, but the increased duration seen with calcium and strophanthin combined persists after 60 min. These facts suggest that calcium and strophanthin are synergic in their action.

Taking into consideration, however, all the changes noted as a result of administration of strophanthin alone and calcium alone in the same subjects, we may conclude that these two agents are not essentially related in their action, in spite of the similar features of their effect.

Fig. 2. E. T. 40-year male. Electrocardiographs (lead II) taken (A) before, (B) 10 min. after, and (C) 60 min. after intravenous injection of 5 c.c. 5 p.c. potassium chloride. Time marker in twenty-fifths of a second.

TABLE II. E. T. 40-year male. Intravenous injection of 5 c.c. 5 p.c. potassium chloride

Serum K concentration mg. per 100 c.c....	Before 20·0	10 min. after 22·8	60 min. after 20·6
	Durations (lead II), sec.		
P	0·12	0·12	0·12
P-R	0·18	0·20	0·20
Q-S	0·10	0·10	0·10
Q-T	0·32	0·36	0·36
T	0·20	0·20	0·20
P-T	0·48	0·52	0·52
T-P	0·16	0·52	0·24
	Heights (lead II), mm.		
P	2	1·5	1·5
R	8	8	8
T	2	2	3

Changes noted after an increase of potassium in the blood serum
(Fig. 2 and Table II)

A lengthening of the duration of the diastolic interval, *T-P*, is the only definite and consistent change which occurs after potassium administration. This change is variable and its extent does not seem to bear any relationship to the proportionate increase of the serum potassium. In many cases the prolongation is not so great as after the administration of calcium. In two cases in which rather larger doses of potassium chloride solution were administered there appeared a remarkable lengthening of the diastole, without any changes in the duration of the other intervals.

With regard to the height of the deflexions, except for one case in which the height of the *P* wave increased from 4 to 5 mm. at 10 min. after the injection and returned to 4 mm. at 60 min. after, in all the rest the height of *P* was diminished. This decrease averages 1 mm. at 10 min. after and in most instances is about the same 60 min. after the injection.

With regard to *R* and *T*, the changes are less constant. In some cases the height of *R* is slightly decreased, in some it remains unaltered, and in others it even showed a slight increase. It may be added that these variations are in no way related to the amount of increase of potassium in the serum. For example, in the three cases which show practically the same percentage increase of potassium after 10 min., the electrocardiograms reveal totally different changes in the heights of *R* and *T*. The height of *T* is sometimes increased.

SUMMARY

1. When calcium or potassium salts are introduced into the blood stream of human subjects, the heart rate is definitely reduced, and this reduction may persist for a considerable time.

2. The electrocardiographic curves obtained have been analysed, and the time durations of the various intervals studied.

3. No definite correlation could be found between the magnitude of the changes in the electrocardiogram and the concentration of calcium or potassium in the blood serum.

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THE EFFECTS OF RESPIRATION UPON THE CIRCULATION THROUGH THE LIVER, AS STUDIED BY MEANS OF X-RAY CINEMATOGRAPHY

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In our last paper [Franklin & Janker, 1936] we described our findings after injection of a contrast medium into the inferior caval systems of the rabbit and of the cat. In the former species there was a suggestion that inspiration facilitated the hepatic inflow into the vena cava and impeded the non-hepatic abdominal inflow into the thorax, while in expiration the reverse occurred. In the latter species the main inflow into the thoracic inferior vena cava, as judged by depth of shadow, occurred in expiration, but a very small extra inflow occurred at the height of inspiration, and we suggested as one possible explanation that this extra inflow might be from the liver. The present experiments were undertaken in order to obtain more information about the effects of respiration upon the hepatic inflow into the vena cava.

Methods

The experiments were performed by us at Bonn upon four cats and one dog under ether anæsthesia. The X-ray cinematographic apparatus used was Janker's animal one [Janker, 1932, 1933], and the animals were photographed in the lateral position. The contrast medium, thorotrast, was injected successively and simultaneously into the right femoral vein, and the splenic or superior mesenteric vein. The cannulæ inserted into the last two veins projected through the reclosed abdominal wall, while the laparotomy was done in such a way as not to weaken the abdominal musculature.

The 35 mm. film record was subjected to examination at Oxford. It was projected a large number of times, many outline drawings were made of enlarged stills, and several photographs were printed from individual frames.

Certain anatomical points were studied during post-mortem dissection of a fifth cat.

Results

In the dog the liver vessels did not show up sufficiently clearly, owing to the thickness of the animal's body, for any useful analysis to be made. In inspiration, however, the "caval band" effect previously noted [Franklin & Janker, 1934] was again evident, and there was also during this phase of respiration a sudden backward thrust of blood in the post-diaphragmatic vena cava.

In the cats the intraportal thorotrast injections did not pass through the small vessels of the liver with sufficient rapidity to show up the hepatic veins and their branches. The opaque medium is said to be taken up by the liver cells as it is by the splenic ones, and this may to some extent account for the finding described. On the other hand, some fluid must have passed through, for the mesenteric and splenic veins were not unduly dilated by the injection, as they can be when there is obstruction to hepatic outflow.

During inspiration there was lengthening, diminution in calibre, and lightening of shadow tone of the thoracic inferior vena cava. The blood passing up the abdominal vena cava, as judged by depth of shadow, was held up distal to the main hepatic vein entries into the vena cava. The shadow of the intrahepatic portion of this vein became narrower in three of the cats; in the fourth there was no change in width of the shadow, but it became tortuous. A similar "concertina" effect was seen in one instance in the mesenteric and portal veins. The liver was compressed, if the changes in the two visible dimensions were paralleled by those in the third; diminutions in length between selected points in the lateral views varied from 3 to 7 p.c. The main hepatic veins became invisible.

During expiration, the thoracic inferior vena cava became shorter and wider, as well as darker from the diaphragm onwards. A backflow from the vena cava into the large hepatic veins near the diaphragm made these vessels visible.

The shadow of the vena cava was indented at the levels of the renal vein entries; the indentation produced at the level of the right renal vein was on the spinal side of the caval shadow, that produced at the level of

the left renal vein was on the opposite side. Examination of the veins *in situ* in a female cat, weighing 3·65 kg., showed the right renal vein entering the vena cava almost at right angles with its inflow directed towards the back, and the left renal vein entering more obliquely, about 1 cm. higher up, with its inflow directed towards the front. During inspiration both renal indentations became more pronounced. No evidence of backflow into the renal veins, or indeed into any of the inferior caval tributaries except lumbar veins, was present in the film.

With the heart and respiration arrested, and during simultaneous intracaval and intraportal injections, there was a backflow from the vena cava into the main hepatic veins. Increase of injection pressures led to great distension of the abdominal vena cava and escape of fluid via lumbar veins into the spinal veins; the thoracic inferior vena cava lengthened and became somewhat S-shaped; the mesenteric and splenic veins, as also the portal vein and its intrahepatic branches, became greatly distended.

Examination of the intrahepatic vena cava of the cat that was dissected showed four large-sized hepatic vein entries, as well as some smaller ones. The first two large ones were at the same level and close to the diaphragm; they, apparently, drained about two-thirds of the liver. The third was situated considerably nearer to the diaphragm than to the caval entry into the liver; the reverse was true of the fourth large entry. If a similar arrangement was present in the cats that were filmed, then only the proximal three large hepatic veins were shown up by the thorotrast; these three between them drain about five-sixths of the liver substance. The angles made by the hepatic vein entries with the long axis of the vena cava were progressively more acute in the dissected cat with increasing distance from the diaphragm.

Discussion

The impossibility of showing up the blood in the hepatic veins by intraportal injections of thorotrast is of great importance, for it means that the hepatic outflow as a whole cannot be made visible by such means. That we were able to detect even a minute inspiratory outflow in our last film [Franklin & Janker, 1936] was probably due to the increased depth of breathing, induced by inhalation of carbon dioxide. Though we showed a preferential outflow from the liver during inspiration, we were misled as to its amount.

In our present experiments, we have reason to assume an inspiratory compression and decrease in volume of the liver. During the expiratory

expansion the hepatic veins doubtless receive extra blood from the small liver vessels, but our results indicate that there is also a tendency for the proximal trunks to fill up from the vena cava. The main hepatic outflow must, therefore, occur during inspiration, parallel with a more or less complete hold-up of the remaining abdominal venous return along the vena cava. The narrowing of the intrahepatic caval shadow during inspiration may be due, as we previously thought [Franklin & Janker, 1934], to actual compression of the vessel; on the other hand, an invisible inspiratory inflow from the more peripheral hepatic veins may be occupying part of the caval space and causing the narrowing of the shadow. We did not realize at that date the extent to which streamlining can occur within the vena cava as a result of tributary inflows [Franklin & McLachlin, 1936 *a*, *b*]. A further possibility, suggested by the study of such streamlining, is that the "caval band" does not restrict the whole of the venous flow from the abdominal into the thoracic inferior vena cava, as it appeared to us to do [see Franklin & Janker, 1934, Figs. 1 and 2], but merely the non-hepatic portion of this flow. The hepatic flow is invisible, but it would presumably occupy that part of the caval lumen which lies farthest from the caval band, for the hepatic vein entries are on this side.

Our earlier views about the inspiratory hold-up of the non-hepatic abdominal flow are corroborated by our present findings. In addition, the greater degree of renal indentation of the caval shadow during inspiration is explicable on the hypothesis that the renal inflows show up better against a slower blood stream within the main vein.

The fact that an invisible stream of blood moves from the liver into the vena cava during inspiration makes necessary a revision of our earlier views about the filling of the thoracic portion of this vessel. On the other hand, our previous statements about the inspiratory tendency of this portion to decrease in calibre were perfectly correct. Inasmuch, however, as the hepatic outflow will tend to increase at the same time as the thoracic inferior vena cava is tending to narrow, the flow along this vein will have to accelerate as inspiration deepens. The necessary condition for this is present, namely, increasing compression of the liver and rising gradient of venous pressure from liver to heart.

The great distensibility of the vessels of the portal system, the hepatic veins, and the abdominal vena cava is worthy of note. If the lumen was circular throughout the pressure rises, many of these veins increased 300 p.c. in cross-section. The distensibility of the vena cava has been given added interest as a result of recent findings [Schretzenmayr, 1936]. The tortuosity that developed in certain veins with high internal

pressures suggests that their transverse distensibility had reached its maximum, and that further response to the pressure was a lengthening; inasmuch, however, as their ends were fixed, this lengthening resulted in distortion.

SUMMARY

In the cat the main hepatic outflow into the vena cava occurs during inspiration, if the liver is compressed.

The English joint author of this paper wishes to express his thanks to Prof. Frhr. v. Redwitz, Director of the University Surgical Clinic, Bonn, as also to Prof. Robert Janker and his assistants in the Radiological Section of the Clinic. The copyright of the film is held by the Reichstelle für den Unterrichtsfilm, Berlin; the authors are indebted to the Reichstelle for providing, free of all charge, the final copy of the film.

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THE ACTION OF ESERINE AND RELATED COMPOUNDS AND OF ACETYLCHOLINE ON THE CENTRAL NERVOUS SYSTEM

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In previous papers [Schweitzer & Wright, 1936, 1937*a*, *b*; Floyd & Keele, 1936] we have examined the effects on the knee jerk and other reflex somatic activities of stimulation of the central end of the vagus, changes in the blood pressure and in the oxygen and CO_2 content of the blood, anæmia of the central nervous system, injection of cyanide, adrenaline and other pressor agents (such as pituitrin and tyramine) and ergotoxin. We have extended our studies and have examined the actions of eserine and related substances and of acetylcholine on the central nervous system with special reference as heretofore to simple somatic reflexes, using the knee jerk as our type reaction. These drugs produce changes in reflex activity which are striking and readily demonstrable by simple means, but in the main have not previously been fully recognized.

Part I. Action of eserine and related substances

Harnack & Witkowski [1876] studied the effects on the nervous system of "physostigmine" (said to be identical with the "eserine" of Duquesnel) and of "calabarin", a second alkaloid claimed to be present in Calabar bean. "Calabarin" was dealt with briefly and was reported to have a strychnine-like action in frogs. "Physostigmine", which was studied more extensively, produced direct paralysis of the central nervous system of frogs in doses of 2–5 mg. In the cat, doses of 1 mg. produced marked initial excitation, "eigenthümliche, zum Theil unmotivierte Bewegungen" and intense fibrillary twitchings of the skeletal musculature. In the guinea-pig the initial excitation was more marked: "die ausserordentlich intensiven fibrillären Zuckungen nehmen hier öfters

einen vollständig rhythmischen Charakter an, und können dem Ungeübten wohl als klonische Krämpfe imponieren". They report an experiment on a human epileptic idiot who was given 0·5 mg. "physostigmine" subcutaneously on three successive days with the result that the frequency of the fits increased enormously. They attributed these stimulating actions of the drug exclusively to the coincident changes in the circulation and respiration. Their general conclusion was that "physostigmine" has a twofold mode of action: "einer *directen Lähmung*...und...einer *directen Erregung*: die erstere ist das *centrale Nervensystem*, die letztere eine, wie es scheint, grosse Anzahl *musculöser Organe*" (italics in the original).

Little systematic work appears to have been carried out on this subject subsequently and the accounts in the principal textbooks (Meyer & Gottlieb, Sollmann, Cushny, Poulsson) follow the report quoted above, even to the extent of saying that the movements produced by eserine "simulate" convulsions. A careful examination of the literature, however, reveals scattered hints that eserine may on the contrary have a central excitatory action. Thus Harnack & Witkowski in one place mention without further comment that "true" convulsions may occur. Rothberger [1901] states that the primary action of physostigmine in the mammal is to excite some part of the central nervous system, probably the cerebral cortex. Heubner [1905] produced "reflex tetanus" in frogs with one preparation of physostigmine as regularly as with strychnine. He also mentions that "epileptiform fits" which were produced with physostigmine in dogs were easily suppressed by chloroform anæsthesia.

Langley & Kato [1915] drew attention to a variety of "spontaneous" movements produced by physostigmine in various species, e.g. irregular contractions and trembling of limbs, clonic movements with either a regular or irregular rhythm, and synchronous nystagmus of both eyes, and conclude that physostigmine in some way causes new impulses to pass out to striated muscle and to certain involuntary structures such as the sweat glands. They state finally that "a small dose of physostigmine has as its primary effect an excitation of a number of centres in the central nervous system. This leaves open the question whether the new nervous impulses are due in part or wholly to increase of excitability of the central synapses".

Henderson & Wilson [1936] injected eserine and acetylcholine into the lateral ventricle in man and found that both drugs produced similar effects; these consisted of nausea, vomiting, increased intestinal movements and sweating, but the circulation and respiration were unaffected.

The actions of both drugs was prevented or abolished by atropine. Sub-stimulating quantities of eserine potentiated the action of subsequently administered acetylcholine.

Methods

The methods we have employed have been essentially the same as those described in our previous communications. Cats were used, usually anæsthetized with chloralose (0·06–0·08 g. per kg. body weight). The knee jerk was recorded in the following manner. The right limb was suitably fixed by means of drills in the femur and ischial tuberosity. The patellar tendon was regularly tapped at intervals of 5, 10 or 30 sec. by an electrically operated automatic hammer, full description of which has previously been given. The movement of the limb was recorded by means of a thread fixed to the lower end of the tibia and connected over pulleys to a Sherrington torsion lever myograph. Simultaneously, in many experiments we studied the response of the quadriceps of the left limb to submaximal or maximal stimulation of the femoral nerve with break shocks using a fluid electrode of the Collison type. The patellar tendon and the quadriceps of the opposite limb were stimulated at the same rate.

Results

Intravenous injection of eserine in doses of 0·1–0·5 mg. (0·03–0·2 mg. per kg. body weight) into cats under chloralose anæsthesia produces an increase in the knee jerk associated with a general heightening of reflex excitability (Fig. 1 A). When bigger doses are employed tonic and clonic movements may develop which are seemingly "spontaneous" in character and will be so referred to, though may possibly be produced reflexly by stray afferent impulses which are constantly being set up. Large doses of eserine in unatropinized animals cause, as is well known, a profound fall of blood pressure owing to an inhibitory action on the heart. These circulatory disturbances may seriously modify the direct effects of the drug on reflex activity. To avoid this complication most of the experiments with large doses of eserine have been carried out after the injection of atropine in amounts adequate to annul the effects of electrical stimulation of the peripheral end of parasympathetic nerves. In such atropinized animals the depressor action of eserine on the blood pressure is considerably diminished, abolished or even reversed. The effects of eserine on reflex activity are not, however, altered in any fundamental respect by this treatment with atropine.

In the account which follows, a description will first be given of those effects produced by eserine which are common to the atropinized and

unatropinized animal. Subsequently the modifications set up by atropine will be considered in detail.

(1) *Increased reflex excitability.* (i) The first effect of eserine is to increase the knee jerk and to heighten general reflex excitability. The

Fig. 1. Cat, 2·8 kg. Chloralose (0·07 g. per kg.). Records from above downwards are knee jerk (right side), contraction of quadriceps (left side) stimulated at intervals of 10 sec. through its motor nerve, blood pressure, time in minutes, signal line. First arrow: inject 0·5 mg. eserine. During period indicated by horizontal white line convulsions took place. Second arrow: inject 1 mg. atropine. Speed up drum momentarily and then at third arrow inject 0·5 mg. eserine. The calibration curve (in g.) for the tension records of this and other figures is shown on the left.

minimum amount of eserine which must be used to stimulate the knee jerk varies considerably with the state of the animal, but in our experience it is about 0·1 mg. (0·03 mg. per kg. body weight). With such threshold doses, there may be a long latent period (up to 3 min. or longer) following which the tension produced by patellar stimulation rises slowly to a maximum after about another 6 min. The knee jerk then slowly declines. When larger doses are used (Fig. 1) the latent period is

progressively shortened, the maximum tension is attained more rapidly, is greater, and is maintained for a longer period at this level. The decline to the pre-injection condition is correspondingly slower. With doses of 0·5 mg. (0·15 mg. per kg. body weight) the tension of the knee jerk at the height of the response may be five or more times as great as initially (Fig. 1), and a period of 20–30 min. or longer may elapse before the knee jerk returns approximately to its original level.

Fig. 2. Cat, 3·2 kg. Chloralose (0·07 g. per kg.). Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. Inject successively 1 mg. ergotoxin, 1 mg. eserine, 2 mg. eserine.

(ii) When the dose of eserine is repeated before the effect of the previous injection has worn off, summation of the response is commonly observed. The tension rises more quickly and to a greater extent, and the results obtained resemble those produced by a single larger dose in the previously untreated or fully recovered animal. If the knee jerk is absent in an animal because of an overdose of anæsthetic or prolonged manipulation, or has been abolished by drugs such as ergotoxin, it can almost invariably be rapidly restored and even markedly enhanced by the injection of appropriate doses of eserine (Fig. 2).

(iii) Not infrequently eserine gives rise to an initial phase of diminished reflex activity which is, however, followed by the increased excitability just described (Fig. 3 A). This initial depression has been mainly observed when large doses, 0·5–2 mg., were employed, especially when given at the height of the response to a previous injection of a large dose of the drug (Fig. 3 B). The initial depression may be of short duration, e.g. a few minutes, but in one instance it lasted for as long as 30 min. after which very intense heightening of the reflex developed. We

Fig. 3. A. Cat, 4·6 kg. Chloralose (0·07 g. per kg.). Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. Inject 2 mg. eserine. This animal had previously received 2 doses of 1 mg. ergotoxin. Note that there is no effect on the blood pressure. B. Cat, 3 kg. Chloralose (0·07 g. per kg.). Previous injection of 2 mg. atropine. Inject 1 mg. eserine. At height of knee jerk response inject 2 mg. eserine. Knee jerk temporarily inhibited.

have been unable to define any more precisely the conditions which influence the appearance of this somewhat uncommon phenomenon of initial depression. We have previously demonstrated [Schweitzer & Wright, 1937*b*] that adrenaline inhibits the knee jerk; it is unlikely, however, that the inhibitory action of eserine is due to the secretion of adrenaline because the amount that has to be injected to produce the effect (about 0·1 mg. per kg. body weight) is far greater than is thought ever to be secreted by the adrenal gland in the body.

(iv) When very large single doses are used or when large doses are repeated in fairly close succession, the phase of increased activity is

followed by a decline to below the normal level, and sometimes the knee jerk may gradually or rapidly disappear completely. In unatropinized animals this secondary failure is often obviously due partly or wholly to the marked depression of the circulation, but the same phenomenon is also observed in atropinized animals in which the blood pressure has not been lowered further to any extent by the drug. This secondary paralysing action of eserine on the central nervous system is the one stressed by previous workers.

(2) *"Spontaneous" movements.* With the bigger doses seemingly spontaneous movements and alterations in tone take place. Sometimes widespread and violent convulsions may occur involving especially the extremities. The changes are not peripheral in origin as they are abolished by section of the nerves to the limb. A graphic record of the frequency and extent of these movements may be obtained quite simply in the following way. The other hindlimb, i.e. the one which is not used for the recording of the knee jerk, is firmly fixed by means of two drills, one in the ischial tuberosity and the other in the lower end of the femur. The leg hangs passively flexed at the knee joint. A thread is tied round the lower end of the tibia and connected under a pulley to a torsion lever myograph. The changes in tension of the unstimulated quadriceps can thus be observed.

Fig. 4 A, B illustrates well the type of "spontaneous" changes in muscle activity which take place and which are best recorded on a more rapidly moving surface. For the sake of brevity these "spontaneous" activities will be referred to as convulsions. The onset of the convulsions may coincide with the increase in the knee jerk, but more commonly it is delayed by some minutes. Their frequency varies a good deal and in marked cases they may be repeated every few seconds. The contraction thus occurring in the quadriceps may exceed that reflexly elicited by patellar stimulation. Sometimes there may be a marked sustained increase in the tone of the quadriceps (e.g. Fig. 3 B), which may be more marked than the increase in the knee jerk. Fig. 4 A shows clonic movements with little change in tone, while Fig. 4 B illustrates a complex combination of tonic and clonic movements. Under these conditions reflex contraction of the contralateral quadriceps sometimes occurs regularly in response to patellar stimulation. It is obvious that these "spontaneous" alterations in quadriceps tension must of necessity modify the knee jerk responses. Firstly, it is known that contracting muscle responds more vigorously to stretching influences, and secondly, there may be a summation of the tension of the convulsion with that of

the reflex contraction. It can, however, frequently be noted that the increase in the knee jerk which occurs in the presence of convulsions is

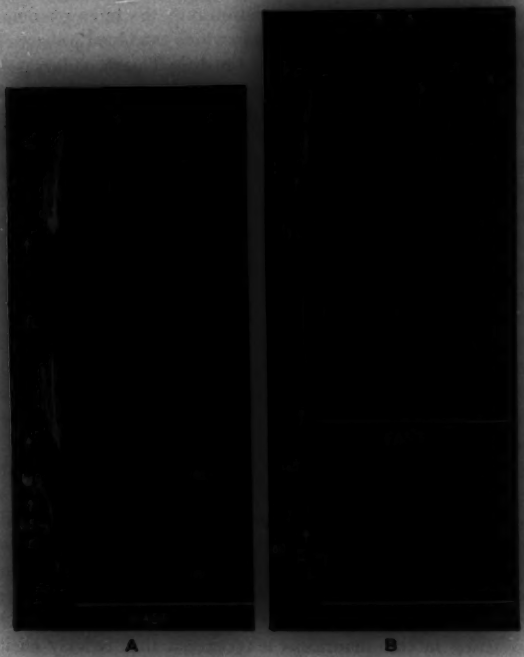


Fig. 4. Records from above downwards are knee jerk (right side), movements of unstimulated hindlimb (left side) (extension is represented by upward movement of lever), blood pressure, time in minutes, signal line. A. Cat, 2·4 kg. Chloralose (0·07 g. per kg.). No atropine. Knee jerk elicited at intervals of 30 sec. Inject 0·5 mg. eserine. B. Cat, 2·4 kg. Chloralose (0·07 g. per kg.). Knee jerk elicited every 10 sec. Inject 2 mg. eserine. Marked tonic and clonic movements. At point indicated by thick white line on signal line change to fast speed of drum. Each *X* on knee jerk records indicates response to patellar stimulation. The other movements are spontaneous.

independent of them and may occur at a moment when no spontaneous changes are in progress (Fig. 4 A). Usually the convulsions die down and disappear before the increase in the knee jerk has passed away.

The following observation bears on the question whether the convulsions are reflexly produced or result from direct stimulation by eserine of the nerve centres. In some experiments the onset of the convulsions occurs during the period of initial depression of reflex excitability as shown by a decrease in the knee jerk. When the convulsions subsequently subside and disappear the knee jerk may progressively increase and finally exceed the pre-injection level. The increase in the knee jerk and the convulsions can, therefore, occur independently one of the other.

Relation to atropine.

As has already been mentioned, atropine does not markedly modify the responses to eserine which have just been described. Atropine in doses sufficient to "paralyse" the parasympathetic terminals, may itself influence the knee jerk. The effects observed vary in intensity in different preparations but there is always a tendency for the knee jerk to be diminished. Not infrequently it is markedly depressed and recovery to the pre-atropine level does not take place. If atropine is injected at the height of the response to eserine, it may produce a transient depression of the knee jerk (Fig. 1) but it does not significantly speed up the rate of decline to the pre-injection level. If an eserine injection is repeated following the administration of atropine our experience is that the response obtained is somewhat less vigorous and the convulsive movements are not so marked (Fig. 1). The difference, however, is not great and it is difficult to attach much significance to these findings as the repeated injection of eserine without previous atropinization may sometimes lead to weaker responses. We have been unable to satisfy ourselves that there is any direct pharmacological antagonism between eserine and atropine in their effects on reflex action, although the effects of atropine are in the reverse direction to those produced by eserine.

Relation to blood pressure.

The excitatory effects produced by eserine are obtained equally well whether the blood pressure is unchanged or raised or lowered (Figs. 1–4). Fig. 3 A shows an initial depression of the knee jerk followed by excitation in an experiment in which the blood pressure was entirely unaffected by the injection of eserine. In atropinized animals doses up to 2 mg. eserine produced no obvious changes in respiration.

The effects produced by eserine are, however, complicated by those of anoxia when the blood pressure is very profoundly lowered in the unatropinized animal, e.g. to 40 mm. or less. As we have previously demonstrated [Schweitzer & Wright, 1937*a*], acute severe anoxia

leads commonly to an increase in quadriceps tone, and after a very transient stimulation of the knee jerk, to a diminution in the reflex response and finally to its complete abolition. The knee jerk may be abolished, however (as previously mentioned), owing to the secondary paralysing action of big doses of eserine on the central nervous system independently of failure of circulation or respiration.

Relation to changes in the response of the quadriceps to nerve stimulation.

In considering the mode of production of the increased reflex responses it is necessary to consider the role of the changes which eserine produces in the muscles themselves. It has been shown by Brown *et al.* [1936] that eserine increases the response of mammalian skeletal muscle to maximal or submaximal motor nerve stimulation. We have in general been able to confirm those findings in animals under chloralose anæsthesia (e.g. Fig. 1). It is obvious that the enhanced responsiveness of the quadriceps muscle to the nerve impulse stream reaching it from the spinal cord must contribute partially to the increase in the knee jerk which is produced by eserine. A number of observations, however, suggest strongly that these peripheral changes, though they play some part, or even a considerable part, do not account wholly for the results obtained. The question was studied by recording the knee jerk in one limb, and in the other the response of the quadriceps to stimulation of its motor nerve with maximal or submaximal break applied at the same rate as that at which the knee jerk was being elicited. Attention may be drawn to the following findings:

(i) The increase in the knee jerk may be produced by eserine in experiments in which practically no increase in the nerve-quadriceps response is obtained.

(ii) The increase in the knee jerk may be proportionately far greater than the increase in the nerve-quadriceps reaction (Fig. 1). Thus in representative experiments the percentage increase in the nerve-quadriceps response was 30, 25, 30, 55, while that of the knee jerk was 600, 320, 80, 100.

(iii) There may be no direct time relationship between the two reactions. Thus in the experiment illustrated in Fig. 5 the knee jerk had practically completely disappeared. Following the injection of 0·3 mg. eserine, the increase in the nerve-quadriceps response occurred after an interval of 3 min., but the knee jerk only began to return after an interval of 10 min. The maximal height of the knee jerk was attained at a time when the nerve-quadriceps responses were declining.

Fig. 5. Cat, 3 kg. Chloralose (0·08 g. per kg.), 5 mg. atropine. Records from above downwards are knee jerk (right side), contractions of quadriceps (left side) stimulated through its motor nerve, blood pressure, time in minutes. Inject 0·3 mg. eserine.

Fig. 6. Cat, 3·6 kg. Decerebrate. Spinal cord divided in the mid-thoracic region. Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. At first arrow inject 1 mg. eserine; between second and fourth arrows induce deep ether anæsthesia; at third arrow inject 1 mg. eserine. The knee jerk is initially stimulated but soon fails partly owing to cessation of respiration resulting from an overdose of the anæsthetic. At fourth arrow discontinue ether and restore breathing by means of a short period of artificial respiration. The knee jerk recovers to a level above that present before the administration of ether. Speed up drum momentarily and at fifth arrow readminister ether; when the knee jerk has practically disappeared, inject at sixth arrow 1 mg. eserine.

(iv) The initial inhibition of the knee jerk which is sometimes obtained with eserine may coincide with a marked increase in the nerve-quadriceps response; the subsequent increase in the knee jerk may take place during the decline of the quadriceps reaction or after it has settled down to its initial condition.

(v) According to Brown *et al.* [1936] the peripheral potentiating action of eserine on the response of skeletal muscle to motor nerve stimulation or to acetylcholine is abolished by deep ether anæsthesia. Fig. 6 shows an experiment where the ether anæsthesia was sufficiently deep almost to abolish the knee jerk and later stopped respiration. Eserine, however, produced the usual stimulating action on the knee jerk.

The conclusion is drawn from all these findings that the increase in the knee jerk produced by eserine is mainly due to an action of the drug on the central nervous system.

Experiments with the hindlimbs separately perfused and with their circulation occluded.

The decisive proof that eserine has a central action can be obtained when the hindlimbs are excluded from the general circulation into which eserine is injected. Under these circumstances eserine can obviously still act on the nerve centres but cannot influence directly the responses of the muscle. We have performed experiments by means of two methods.

(i) We have blocked the lowest part of the abdominal aorta and the inferior vena cava using strong intestinal clamps, thus depriving the hindlimbs of almost the whole of their circulation. We have previously shown that in such ischæmic hindlimbs the knee jerk can be elicited for periods up to 20 min. without serious impairment. In such preparations the injection of eserine into the jugular vein produces the usual increase in the knee jerk (Fig. 7). Convulsive movements may also be observed.

In some experiments more elaborate methods were employed to diminish the possibilities of any collateral circulation being established by tying in addition the common iliac and femoral arteries on both sides, but this did not annul or modify the stimulatory action of eserine on the knee jerk.

(ii) The other method employed was to perfuse the hindlimbs by means of the Dale-Schuster pump, using warm oxygenated Ringer-Locke solution or the weak hæmoglobin-Ringer solution recommended by Amberson & Höber [1932–3]. The perfusion was carried out through cannulæ inserted into the lowest part of the abdominal aorta and the inferior vena cava. In our hands the reaction of the quadriceps to

patellar stimulation rapidly deteriorated under these conditions and usually disappeared in about 20–30 min., presumably because of the changes in the state of the muscle; gross œdema was obvious. In such preparations, however, the injection of eserine into the jugular vein still caused a transient marked increase in the knee jerk. This could be obtained even when the knee jerk had previously completely disappeared (Fig. 8). In some experiments, when the knee jerk had disappeared, a

Fig. 7.

Fig. 8.

Fig. 7. Cat, 2·6 kg. Chloralose (0·08 g. per kg.), 5 mg. atropine. Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. At arrow, clamp lower part of abdominal aorta and inferior vena cava and then inject 3 mg. eserine.

Fig. 8. Cat, 3 kg. Chloralose (0·08 g. per kg.), 5 mg. atropine. Records from above downwards are knee jerk, time in minutes, signal line. Hindlimbs perfused with warm oxygenated Ringer-Locke solution by means of cannulæ in lower part of abdominal aorta and inferior vena cava. Inject successively 3 mg. eserine into the jugular vein, 3 mg. eserine into peripheral circulation.

comparison was made between the effects of eserine injected into the jugular vein and of the same dose introduced into the perfusion system so as to reach the hindlimbs only. The peripheral injection was without effect on the knee jerk, while the jugular injection gave the usually excitatory response. The same result was obtained if the order of the injections was reversed (Fig. 8).

In both series of animals described in this section methylene blue was injected into the jugular vein at the end of the experiment and sections of the hindlimb tissues were subsequently examined macroscopically and microscopically, but showed no trace of the dye. It can be concluded, therefore, that the procedures employed were adequate in cutting off the hindlimbs almost completely from the circulation in the animal as a whole, and that the eserine injected into the jugular vein produced its effect solely by an action on the central nervous system and not by any peripheral action on the quadriceps muscle. The conclusion previously arrived at is confirmed, therefore, that eserine has a direct action on the central nervous system increasing reflex excitability and giving rise to "spontaneous" changes in tone and movement.

Relation of twitching to increased reflex activity.

As many previous workers have shown [see Langley & Kato, 1915], eserine produces muscular twitchings which may be marked and generalized. These movements are mainly if not wholly peripheral in origin; they are readily produced in a muscle following acute section of its motor nerve, are unaffected by atropine but are abolished by curare. It might be argued that the afferent impulses set up in the muscles by twitchings contribute to the heightening of reflex responses in general and of the knee jerk in particular. This possibility is not wholly excluded by the perfusion experiments recorded in the foregoing section, for following an injection of eserine into the jugular vein twitching still takes place in the forepart of the body and this may reflexly influence the activity of the lumbar spinal cord and so enhance the knee jerk. Against this view it may be pointed out:

(i) An increase in the knee jerk may occur with doses of eserine too small to produce detectable twitching.

(ii) Muscle tone (like the knee jerk) is the result of a stretch reflex, but doses of eserine which set up marked twitching may produce no change in the tone of the quadriceps muscle (which is not subjected to patellar stimulation), although the knee jerk is enhanced.

(iii) An experiment was performed as follows: the lower part of the abdominal aorta and inferior vena cava were occluded and curare was injected into the jugular vein in sufficient amounts to paralyse the forepart of the animal. Eserine injected into the jugular vein produced no twitching anywhere in the animal but the knee jerk was increased in the characteristic way.

It seems unlikely, therefore, that the twitching is concerned to any significant extent in heightening reflex excitability.

Observations in the spinal animal.

(i) The increase in the knee jerk produced by eserine is seen equally well in animals under chloralose anæsthesia in which the spinal cord has been divided in the mid-thoracic region. The drug, therefore, acts directly on the spinal centres. Convulsions have also been noted in such preparations. Enough experiments have not yet been carried out to decide whether the convulsions are as readily elicited in the spinal as in the intact animal.

(ii) The effects of eserine are also strikingly seen in decerebrate animals which subsequently had their spinal cord divided and which were allowed to rest long enough for the effects of the initial ether anæsthesia to wear off (Fig. 6). The excitatory effects of eserine are, therefore, not due to any anti-anæsthetic action.

Fig. 9. Cat, 3·3 kg. Chloralose (0·07 g. per kg.). Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. A. Inject successively 0·5 mg. prostigmine, 1 mg. prostigmine, .3 mg. atropine, 1 mg. eserine. Interval of 5 min. between A and B. B. Inject successively 1 mg. eserine, 1 mg. prostigmine, 1 mg. prostigmine, 1 mg. eserine.

Action of prostigmine and Stedman's "meta" compound.

(i) Prostigmine increases the response of skeletal muscle to motor nerve stimulation [Wilson & Wright, 1936]; its peripheral potentiating action on skeletal muscle is probably greater than that of eserine. This

effect is due to its anti-cholinesterase action. When injected into unatropinized animals in doses of 0·5 mg. prostigmine slightly depresses the knee jerk, and may completely abolish it for many minutes in doses of 1 mg. (Fig. 9). These actions are not annulled by atropine either given prior to the injection of prostigmine or during the period of inhibition

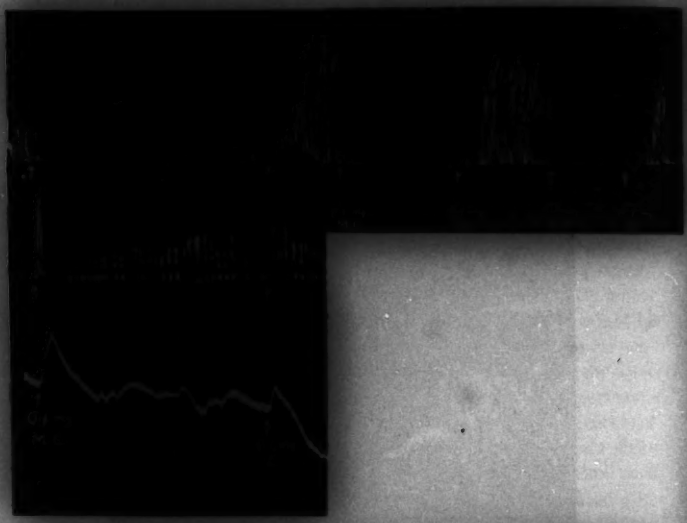
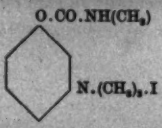


Fig. 10. Cat, 3·5 kg. Chloralose (0·07 g. per kg.), 3 mg. atropine. Records from above downwards are knee jerk (right side), contractions of quadriceps (left side) stimulated through its motor nerve at intervals of 10 sec., blood pressure, time in minutes, signal line. Inject successively 0·1 mg. "meta" compound, 0·1 mg. eserine, 0·1 mg. "meta" compound, 0·1 mg. eserine, 0·1 mg. "meta" compound, 0·1 mg. eserine.

set up by the drug. The inhibition of the knee jerk occurs equally well whether the blood pressure is lowered or raised by prostigmine. It is independent of changes in respiration. As the peripheral action of prostigmine on muscle is excitatory there can be no doubt from the above results that prostigmine exerts a direct inhibitory influence on the central nervous system. The injection of eserine during the period of central inhibition set up by prostigmine gives rise to the usual excitation manifested by a marked increase in the knee jerk above the normal level, and

by convulsions. Prostigmine given subsequently to an injection of eserine may diminish or abolish the excitatory effects of the latter drug.

(ii) Dr Stedman has very kindly put at our disposal a small sample of a new member of the eserine series which he has prepared. This "meta" compound (as he calls it for brevity) is the methylcarbamic ester of *m*-hydroxyphenyltrimethyl ammonium iodide and has the formula



From the results of unpublished experiments Dr Stedman believes that it is the most effective known inhibitor of cholinesterase. According to Bacq & Brown [1937] the "meta" compound has a far greater (about tenfold) action than eserine in potentiating the effects of motor nerve stimulation on skeletal muscle. We have examined the action of the "meta" compound on the knee jerk and find that it has a central inhibitory action identical with that produced by prostigmine. It is effective, however, in far smaller doses—e.g. 0·1 mg. of the "meta" compound will abolish the knee jerk as compared with about 1·0 mg. of prostigmine (Fig. 10). The central inhibitory action of the "meta" compound is unaffected by atropine but may be overcome by eserine.

Action of pilocarpine and arecoline.

Sollmann [1932] states that pilocarpine is mainly depressant to the central nervous system, but that the motor centres, especially those of the spinal cord, are stimulated giving rise to increased reflexes, tremors, convulsions; finally paralysis sets in. Pilocarpine injected into the lateral ventricles (cat) induces excitement [Dikshit, 1935].

In our hands pilocarpine injected intravenously in doses of 1–5 mg. has produced convulsions with little or no stimulating action on the knee jerk. We have obtained similar results with arecoline.

Part II. Action of acetylcholine

Dikshit appears to have been the first worker to examine carefully the action of acetylcholine on the central nervous system. He showed [1934, 1935] that when it was injected into the cerebral ventricles (cat) respiration was temporarily inhibited and a condition produced resembling

natural sleep. Silver & Morton [1936] produced drowsiness in unanæsthetized cats by intracisternal injection of acetylcholine. Reference was made in Part I to the observations of Henderson & Wilson [1936] in man. Suh *et al.* [1936] found that intracisternal injection of acetylcholine in the cat raised the blood pressure and stimulated respiration. Coombs & Cope [1936] report that acetylcholine arrests the clonic convulsions induced experimentally in cats by means of camphor monobromide, but the restlessness, struggling, deep gasping or rapid respiration and tonic extensor movements persist. Carmichael & Fraser [1931–3] in their clinical study of the actions of acetylcholine in man make no reference to effects on the nervous system. Feldberg & Schriever [1936] showed that following the injection of adrenaline in the eserinized animal acetylcholine appeared in the cerebrospinal fluid. Choline compounds are said to be present normally in the cerebrospinal fluid in man, the amounts found [Page & Schmidt, 1931] in three subjects being 0·09–0·37 mg. per l.; the concentration is altered in various diseases. Schweitzer & Wright [1937 *a*, *b*] did not succeed in showing that acetylcholine was concerned as an intermediary when central vagus stimulation or the injection of adrenaline inhibited the knee jerk. Acetylcholine can be extracted from various parts of the central nervous system [Chang & Gaddum, 1933; Dikshit, 1934; Barsoum, 1935; Kwiatkowski, 1935]. According to Quastel *et al.* [1936], when brain cortex slices are allowed to respire in a medium containing eserine, a choline-ester (possibly acetylcholine) is formed. Its formation cannot be demonstrated in the absence of eserine. The choline ester is regarded as being formed as a result of the metabolic processes in the tissue slice, only a small fraction of it being due to preformed ester within the tissue. Eserine alone did not appear to affect the respiration of the brain slice. Acetylcholine dilates cerebral blood vessels [Wolff, 1929] and has been employed without success in the treatment of epilepsy in man [Lloyd, 1933; McLaughlin, 1933].

Results

Experiments in animals without atropine.

The intravenous injection of acetylcholine in doses of 0·1 mg. (0·03 mg. per kg. body weight) or over usually depresses the knee jerk (Fig. 11). The effect as a rule sets in rapidly, sometimes within 20–30 sec. after the moment of injection. The inhibition attains its maximum level quickly (in 1 or 2 min.) and then passes off more gradually. Sometimes after the initial sharp decline there is a secondary slow dwindling of the knee jerk

which may even disappear completely in 10 or more minutes after the injection. This is followed by a gradual and possibly partial recovery. With larger doses of acetylcholine the inhibition sets in after a shorter latent period, its extent is greater and recovery takes place more gradually.

Not uncommonly there is also a gradual rise in the base-line of the knee jerk record which probably indicates an increase in quadriceps tone.

Fig. 11. Cat, 3 kg. Chloralose (0·07 g. per kg.). Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. At first signal stimulate peripheral end of vagus, coil at 5. At first arrow inject 0·2 mg. acetylcholine, at second arrow inject 1 mg. atropine. At fourth and fifth signals stimulate peripheral end of vagus, coil at 5 and coil at 0. No effect. At third, fourth, fifth and sixth arrows inject successively 0·2 mg. acetylcholine, 1 mg. acetylcholine, 2 mg. eserine, 1 mg. acetylcholine.

Its rate of development is somewhat variable. It may attain its maximum at about the time of greatest depression of the knee jerk, or it may be delayed for some little time after that. Following this increase in tone, a secondary decrease in tone may take place which coincides with the recovery of the knee jerk. The diminution in tone is often quite gross and shows itself by an obvious slackening of the thread connecting the leg to the myograph lever and the knee jerk may become pendulum in character. When the blood pressure is lowered by acetylcholine to 40 mm. or lower there may be an initial transient stimulation of the knee jerk; this

is probably due, as our previous studies have shown, to anæmia of the nervous system. The transient increase in tone referred to above may perhaps also be due, partly at any rate, to the same cause.

When the response of the quadriceps on the opposite side to stimulation of its motor nerve is simultaneously recorded, it is found to be unaffected by these doses of acetylcholine. The effects described, therefore, must be due directly or indirectly to an action of acetylcholine on the central nervous system.

It is improbable that the inhibition of the knee jerk is due solely to the changes in the circulation, though these as suggested above probably play a part. As acetylcholine markedly dilates the cerebral blood vessels the diminution in blood flow through the nervous system is probably less severe than the fall of blood pressure alone would indicate. We have previously demonstrated that when the blood pressure is lowered by hæmorrhage by less than 100 mm. or to a level of about 40 or 50 mm., the knee jerk is unaffected or if altered at all shows an increase in amplitude. We have frequently observed an inhibition of the knee jerk by acetylcholine in unatropinized animals when the blood pressure has been lowered to a smaller extent than 100 mm. and to a level not below, e.g. 70 mm. It will be shown in the next section that acetylcholine inhibits the knee jerk when the fall of blood pressure is prevented or reversed by the previous administration of atropine.

Action of acetylcholine in atropinized animals.

The study of the action of acetylcholine after atropine is complicated by the fact already referred to that atropine itself in the requisite doses tends very commonly to depress the knee jerk. In animals in which the atropine did not seriously diminish the knee jerk a study was made of the effects of acetylcholine before and after atropinization. It was generally found that after atropine acetylcholine produces less marked effects on the knee jerk. Small doses of the drug (e.g. 0·1 mg.) which produced marked inhibition of the knee jerk in the unatropinized animal may have their effect completely annulled by atropine (Fig. 11). In some experiments (Fig. 12) atropine did not completely abolish the central inhibitory action of acetylcholine but the depression of the knee jerk set in after a longer latent period (e.g. 5 min.), maximum inhibition was attained more gradually (e.g. after another 7 min.) and recovery was more rapid. When the effects of small doses of acetylcholine are completely annulled by atropine, larger doses may still produce a considerable degree of inhibition. The abolition by atropine of the vascular

and nervous effects of acetylcholine does not always take place to the same extent. Thus the inhibitory action of acetylcholine on the knee jerk may be abolished by amounts of atropine which do not wholly annul the muscarine action on the heart or the fall of blood pressure. Conversely atropine may reverse the depressor action of acetylcholine on the blood pressure without annulling the inhibition of the knee jerk. Exceptionally the central action of acetylcholine is unaffected by the administration of atropine. When acetylcholine abolishes the knee jerk in the unatropinized animal the subsequent injection of atropine does not facilitate its return.

Fig. 12. Cat, 3·7 kg. Chloralose (0·08 g. per kg.), 5 mg. atropine. Records from above downwards are knee jerk (right side), contractions of quadriceps (left side) stimulated through its motor nerve, blood pressure, time in minutes, signal line. Inject successively 1 mg. acetylcholine, 5 mg. acetylcholine.

The experiments in the atropinized animal prove conclusively that the central action of acetylcholine is not related to or dependent on the direction or degree of change in the blood pressure.

After atropine it is possible to study the effects of bigger doses of acetylcholine which could not be employed in the unatropinized animal owing to the circulatory disturbance which they produce. These big doses, e.g. 5 mg. or over, produce a long-lasting and often a permanent abolition of the knee jerk (Fig. 12).

The response of the femoral nerve-quadriceps preparation was studied together with the knee jerk in atropinized animals. With doses of acetyl-

choline of less than 1 mg. no change is observed in the quadriceps response even when a substantial decrease in the knee jerk was produced. With doses of 1 mg. or over the reaction of the quadriceps is sometimes modified and more regularly when still bigger doses, e.g. 5 or 10 mg. or over are used. The type of change observed is quite constant (Fig. 12). There is an initial small increase in the resting tension level of the quadriceps which presumably represents a contracture. This is associated with a diminution in the height of the knee jerk response which may sometimes disappear altogether. This diminished responsiveness of the muscle to motor-nerve stimulation (curariform action) usually long outlasts the short-lived contracture. This phenomenon will be referred to more fully later. If the quadriceps reaction and the knee jerk are compared it is usually found that during the period of maximum depression of the knee jerk the quadriceps may have already recovered completely to its normal state (Fig. 12). There can be no doubt, therefore, that after atropine also, the depression of the knee jerk produced by acetylcholine is almost entirely of central origin.

Large doses of acetylcholine also produce twitchings resembling those set up by eserine and like them they persist after section of the motor nerve to the muscle. The quadriceps may show vigorous fibrillary twitching when its response to patellar stimulation has been completely abolished by acetylcholine.

Striking changes also occur in respiration. With doses of acetylcholine of the order of 10–25 mg., there is an initial intense spasm or contracture (we are unable to say which) of the diaphragm on which small respiratory movements may be superimposed. This is followed by a return of the diaphragm to its normal expiratory position. At this stage the breathing movements are still absent or greatly reduced in amplitude. There may be then a secondary slow rise of expiratory tension with progressive recovery in the extent of the respiratory movements which may become markedly increased above the normal. This usually occurs about the time when expiratory tone has returned to its pre-injection state. Stimulation of respiration continues for many minutes, during which period the knee jerk is completely absent.

Heymans *et al.* [1936] have shown that acetylcholine reflexly stimulates respiration by acting on the vaso-sensory zones, but do not exclude the possibility that it may also act on the respiratory centre directly.

Action of acetylcholine after eserine.

In atropinized animals the inhibitory action of acetylcholine on the knee jerk is often considerably potentiated by doses of eserine (e.g. 0·5–2 mg.), which markedly increase the knee jerk. Thus in the experiment illustrated by Fig. 11, 1 mg. acetylcholine was without effect prior

Fig. 13. Cat, 3 kg. Chloralose (0·08 g. per kg.). Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. A. Inject successively 2 mg. atropine, 0·2 mg. acetylcholine, 1 mg. acetylcholine. During interval between A and B, inject another 3 mg. atropine which diminishes the knee jerk. B. Inject 2 mg. eserine, speed up drum momentarily and then inject successively 0·05 mg. acetylcholine, 0·2 mg. acetylcholine.

to the administration of eserine but produced marked inhibition subsequently. In another experiment 0·2 mg. acetylcholine had no effect on the knee jerk and 1 mg. produced only a slight transient contracture (Fig. 13 A); after eserine, however (Fig. 13 B), 0·05 mg. acetylcholine produced a contracture and a slight inhibition of the knee jerk, 0·2 mg. a marked contracture and a substantial decrease in the knee jerk, while 1 mg. (not illustrated) completely abolished the knee jerk. The twitching movements are also intensified by eserine.

In an attempt to analyse these results a number of factors must be taken into account.

(i) Eserine intensely stimulates the spinal centres and so sets up a more difficult background which must be overcome by acetylcholine before it can produce its inhibitory effects. The central action of eserine thus directly antagonizes the central action of acetylcholine.

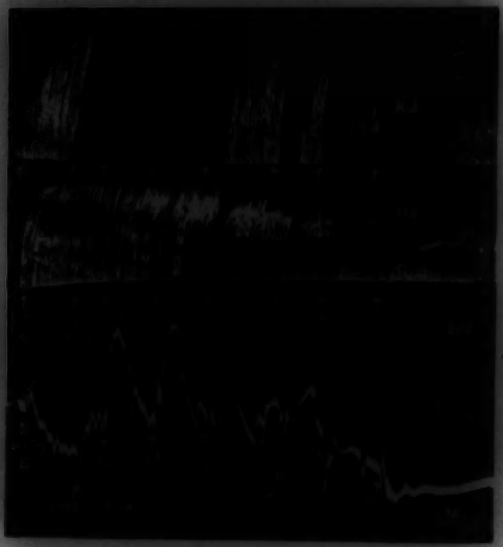


Fig. 14. Cat, 3 kg. Chloralose (0·07 g. per kg.), 5 mg. atropine. Records from above downwards are knee jerk (right side), contractions of quadriceps (left side) stimulated through its motor nerve at intervals of 10 sec., blood pressure, time in minutes, signal line. Inject 2 mg. eserine; then inject successively doses of acetylcholine as follows: 0·05, 0·2, 0·5, 1·0, 2·0, 5·0, 10·0 mg.

(ii) Eserine inhibits the action of cholinesterase [Ammon, 1935] which is known to be present in relatively large amounts in the central nervous system [Stedman & Stedman, 1935]. This may be supposed to enable injected acetylcholine to persist longer and act more vigorously on the nerve centres. In this way eserine would potentiate the central inhibitory action of acetylcholine.

(iii) The curariform action of acetylcholine on skeletal muscle is well marked in eserinized animals [Rosenblueth *et al.* 1936]. We have found that following the injection of eserine the curariform action of acetylcholine is markedly potentiated; doses of acetylcholine of 0·2–1 mg. may be as effective after eserine as 10–20 mg. previously. This peripheral action of eserine also favours the potentiation of the inhibitory effects of acetylcholine.

Fig. 15. Cat, 3 kg. Chloralose (0·03 g. per kg.), 2 mg. atropine, followed by 2 mg. eserine. Records from above downwards are knee jerk, blood pressure, time in minutes, signal line. Inject successive doses of acetylcholine as follows: 0·02 mg. (pure stimulation of knee jerk), 0·5 mg. (slight stimulation of knee jerk followed by depression), 1 mg. (pure depression of knee jerk), 5 mg. (stimulation of knee jerk followed by depression).

It can be shown, however, that potentiation of the central action of acetylcholine is a factor in the total result obtained following the injection of eserine. The experiment from which Fig. 14 is taken shows that in the eserinized animal complete inhibition of the knee jerk may still be present when the response of the quadriceps to nerve stimulation has returned to its original level.

In atropinized animals and more especially after eserine, evidence may sometimes be obtained that the central action of acetylcholine is not wholly one of inhibition, but that an excitatory component is present

as well (Fig. 15). Excitation may occasionally be the only effect of small doses (e.g. 0·02 mg.) of acetylcholine, and larger doses (1–5 mg.) may produce an initial excitation followed by the usual inhibition.

In the eserinized animal also there is no relationship between the effects produced by acetylcholine on the blood pressure and on the knee jerk (Figs. 14, 15).

Fig. 16. Cat, 2·6 kg. Chloralose (0·08 g. per kg.), 5 mg. atropine followed by 3 mg. eserine. Records from above downwards are knee jerk, blood pressure, time in minutes. At first arrow, clamp lower part of abdominal aorta and inferior vena cava. At second and third arrows inject successively 0·2 mg. acetylcholine, 0·5 mg. acetylcholine. At fourth arrow release aorta and inferior vena cava. At fifth arrow inject 0·5 mg. acetylcholine.

Action in spinal animal.

Acetylcholine inhibits the knee jerk in the spinal animal so that it doubtless acts directly on the nervous elements in the lumbar cord.

Central action of acetylcholine.

Further evidence is provided that acetylcholine inhibits the knee jerk by an action on the cord by the use of the methods described in Part I

which enable one to isolate the circulation in the hindlimbs from that to the nervous system. When the hindlimbs are perfused with oxygenated Ringer-Locke solution or Amberson-Höber weak hæmoglobin-saline, acetylcholine injected into the jugular vein depresses the knee jerk. The inhibition produced is sometimes greater when the dose is repeated following an injection of eserine, thus giving additional support to the view that eserine potentiates the central action of acetylcholine. Similar results are obtained if acetylcholine is injected into the jugular vein when the circulation to the hindlimbs is occluded. In the experiment from which Fig. 16 is taken the inhibition of the knee jerk was approximately the same whether the circulation to the hindlimbs was intact or arrested.

Anti-strychnine action of prostigmine, the "meta" compound and acetylcholine.

Preliminary experiments (which will be more fully reported later) show that the convulsions induced by strychnine (in doses of 0·1–0·2 mg. per kg. body weight) can be markedly reduced or temporarily abolished by appropriate amounts of prostigmine, the "meta" compound or acetylcholine. Following the injection of prostigmine the anti-strychnine action of acetylcholine is markedly enhanced. If injections are made in close succession of prostigmine, acetylcholine and strychnine in the order named, the onset of the strychnine convulsions may be prevented wholly for periods up to 10 min. or longer.

Discussion

We have demonstrated that acetylcholine inhibits excitatory somatic reflexes by a direct action on the spinal centres which is independent of changes in the circulation, respiration or the responsiveness of the muscles themselves. We are unable at present to explain more fully the mode of action of acetylcholine, but two suggestions may be put forward tentatively for subsequent experimental analysis:

(i) It may act on the afferent side of the reflex arc preventing the formation of the central excitatory agent or hindering its access to the anterior horn cells.

(ii) It may exert a direct paralysing action on the anterior horn cells so that they are unable to discharge.

The experiments recorded above prove that eserine has a direct and striking action on the spinal cord. The changes in the reflex responses described are not due to alterations in the circulation or respiration; the peripheral stimulating action of eserine on skeletal muscle is probably

only of minor importance in causing the increase in reflex activity which has been observed. It may be suggested that eserine, like strychnine, facilitates the transmission of excitatory afferent impulses through the synapses in the spinal cord possibly by stimulating the formation or prolonging the duration of action of the hypothetical central excitatory substance, state, or "transmitter". Alternatively, or in addition, eserine may possibly have a "ganglionic" action, i.e. it may act on the anterior horn cells causing them to discharge, or facilitating their response to the synaptic transmitter. The so-called "spontaneous" movements produced by eserine may be due to a "ganglionic action" or they may simply be a manifestation of the "strychnine" action, i.e. of generally heightened reflex excitability. We cannot altogether dismiss the former view as we are especially impressed by the fact that convulsions may occur at a time when reflex excitability, as judged by the height of the knee jerk, is depressed.

It is next necessary to consider to what extent eserine stimulates the spinal cord by virtue of its power of inhibiting the action of the cholinesterase which is undoubtedly present in the central nervous system. This question must be answered in the light of the central action of acetylcholine itself and of the other members of the eserine group. If the central effects of eserine are due solely to inhibition of cholinesterase then eserine and acetylcholine should produce similar changes in the central nervous system. This, as our work has shown, is undoubtedly not the case: the main central action of eserine is excitatory and that of acetylcholine is inhibitory. Eserine, however, seems to inhibit the cholinesterase in the nerve centres *in vitro* [Quastel *et al.* 1936] and in conformity with this we find that it potentiates the central inhibitory action of acetylcholine on the spinal reflexes. The discrepancy between the central actions of eserine and acetylcholine can be explained by assuming that eserine has a dual central action due probably to different chemical groupings within the molecule: it inhibits cholinesterase and so tends (like acetylcholine) to inhibit reflexes, and in addition directly stimulates certain excitatory elements or agencies in the spinal cord ("strychnine-like" action). Reference has been made in the text to the fact that eserine sometimes produces an initial inhibition of reflexes: this might be explained by the occasional predominance of its anti-cholinesterase action which is usually masked by the more outstanding central excitatory action.

This general conclusion is supported by the results obtained with other members of the eserine group. Prostigmine probably, and the "meta"

compound certainly, have a greater (the latter a far greater) inhibitory action than eserine on cholinesterase in skeletal muscle. Possibly they also have a more powerful anti-cholinesterase action in the spinal cord. Stedman has found that his "meta" compound is the most potent known inhibitor of cholinesterase *in vitro*. This line of reasoning suggests that their central action on reflexes should be inhibitory, i.e. resemble that of acetylcholine; this in fact has been shown to be the case. More numerous experiments will have to be performed, however, to decide whether they are absolutely devoid of any central excitatory action.

Our experiments with strychnine also support the thesis so far outlined. It is universally admitted that strychnine convulsions are due to a central excitatory action of the drug. These convulsions are abolished by prostigmine and the "meta" compound—drugs which have a peripheral excitatory action on skeletal muscle, and by acetylcholine in doses which have little or no peripheral depressant action on muscle. These drugs must therefore produce some form of central inhibition.

If the central inhibition produced by prostigmine and the "meta" compound is rightly attributed (even only in part) to an anti-cholinesterase action, then judging from the analogy of peripheral organs one would be tempted to suggest that acetylcholine may be readily available in the normal nervous system in a form capable of producing physiological effects. This view is supported by the recent work of Quastel *et al.* [1936] who found a choline ester formed by slices of brain cortex when the medium contained eserine.

Pilocarpine and arecoline according to Stedman & Stedman [1931] have no anti-cholinesterase action. The former is generally described as a pure convulsant and that has been our experience with both drugs.

Many (but not all) physiological actions that are mediated by acetylcholine are annulled by atropine; the same is the case for many of the effects of injected acetylcholine. Our experiments, however, show that atropine has little influence on the central inhibitory action of prostigmine or of the "meta" compound (though we attribute their action (in part at any rate) to the intervention of acetylcholine). Langley & Kato [1915] claimed that all the contractions in striated muscle produced by physostigmine which they considered to be of central origin may be decreased or stopped by a few milligrams of atropine. Our evidence is, however, to the contrary. Though our general impression is that the excitatory effects of eserine are slightly reduced in the atropinized animal (Fig. 1) we have conclusively demonstrated that eserine can still produce marked central excitation even following the injection

of atropine in large doses (5–10 mg., i.e. 2–3 mg. per kg.). Atropine, however, is more effective in annulling partially or wholly the central inhibitory action of small doses of acetylcholine itself. In general it is probably unwise to draw conclusions too readily from the results of these complex drug interactions.

We have emphasized in the text that we are not wholly satisfied that the central action of acetylcholine is purely inhibitory and suspect that in some of our experiments an excitatory component may have been present as well.

It is important to stress that we have only studied the effects of this group of drugs on excitatory somatic reflexes. We have yet to determine whether they can modify inhibitory somatic reflexes.

All the pharmacological actions of the eserine group and of acetylcholine are deeply interesting because of the proved relationship of these drugs (mainly by Loewi, Dale and their co-workers) to transmission of the nerve impulse in the peripheral autonomic nervous system and in motor nerves to skeletal muscle. (For review of literature see Dale [1934], Loewi [1935], and Gaddum [1935].) There has naturally been much speculation concerning the possible existence, identity and role of chemical transmitters in the central nervous system, but experimental work on the subject is scanty. Our results prove that members of the eserine group and acetylcholine itself can profoundly modify transmission processes in the spinal cord. We have referred to chemical work showing that cholinesterase is present in the central nervous system and that a choline ester can be liberated by respiring slices of cerebral cortex. The evidence now available points to the possibility that acetylcholine or a similar substance may be normally intimately related to the process of central transmission of somatic reflexes. But even if this conclusion is agreed to it does not necessarily follow that such naturally released acetylcholine must function as a depressing or inhibiting agent. The method of intravenous injection employed in this research mimics very imperfectly the conditions under which acetylcholine might be formed naturally at central synapsis. In the case of many drugs the route of introduction and the dose employed profoundly influence not only the extent but even the direction of the effect. The action of acetylcholine itself provides a salutary warning. Under certain conditions it produces a vigorous repetitive muscular contraction, while under slightly different circumstances it may give rise to the exactly opposite effect, namely a curari-like paralysis [Rosenblueth *et al.* 1936; Brown *et al.* 1936, Fig. 14 (*supra*)].

The results with prostigmine and the "meta" compound should perhaps carry more weight in indicating the direction of action of (hypothetically) naturally released acetylcholine in the central nervous system. The intensity with which these drugs mimic the central action of acetylcholine and inhibit somatic reflexes runs roughly parallel to their known anti-cholinesterase action *in vitro* and on skeletal muscle. But even here a complicating factor cannot as yet be excluded, namely that prostigmine and the "meta" compound may inhibit reflexes in part owing to the specific action of a chemical grouping other than that which is responsible for the anti-cholinesterase action.

Summary and conclusions

1. The action of eserine and related substances and of acetylcholine on the central nervous system has been studied in cats (usually under chloralose anæsthesia), using the knee jerk as the type somatic reflex.

2. Eserine (0·03–0·2 mg. per kg. body weight) markedly increases the knee jerk and general reflex excitability. Larger doses (up to 0·75 mg. per kg.) produce an increase in tone and convulsive movements. Occasionally initial inhibition precedes the onset of the phase of excitation.

3. Atropine (0·5–2 mg. per kg.) commonly depresses the knee jerk owing to a central action; it does not antagonize to any marked extent the excitatory action of eserine on reflex activity.

4. Detailed analysis has shown that the excitatory action of eserine on reflexes is not due to changes in the blood pressure or respiration and is only partially dependent on the increased responsiveness of skeletal muscle. Eserine produces excitation in animals under deep ether anæsthesia which abolishes the peripheral potentiating action of eserine on skeletal muscle.

5. Experiments with the hindlimbs separately perfused or with their circulation occluded prove that eserine acts directly on the central nervous system. Eserine acts equally well in the decerebrate animal (proving that its action is not merely anti-anæsthetic) and after mid-thoracic spinal transection, so that it influences the knee jerk by a direct action on the lower spinal somatic centres.

6. Prostigmine and Stedman's "meta" compound depress the knee jerk owing to a direct inhibitory action on the spinal cord which is independent of changes in circulation and respiration and is unaffected by atropine. The inhibition is abolished by suitable doses of eserine.

7. Prostigmine, acetylcholine and the "meta" compound can abolish convulsions induced by strychnine.

8. Pilocarpine and arecoline are purely convulsant drugs and have little heightening action on the knee jerk.

9. Acetylcholine (0·03 mg. per kg. or over) usually depresses the knee jerk in unatropinized animals. The effects of small doses are annulled by atropine, but larger doses of acetylcholine may still produce their usual effect. In the atropinized animal the inhibition set up is central in origin and is independent of changes in circulation, respiration or in the responsiveness of the muscle to motor-nerve stimulation.

10. Eserine increases the inhibitory effects of acetylcholine on the knee jerk. This is due partly to potentiation of the peripheral curariform action of acetylcholine, but mainly to an intensification of its central inhibitory action.

11. Acetylcholine inhibits the knee jerk in the spinal animal and when the hindlimbs are separately perfused or have their circulation occluded.

12. The central excitatory action of eserine cannot be due to its anti-cholinesterase action as it is the converse of the central action of acetylcholine. It is attributed to a direct action on the spinal cord which may be "strychnine-like" (facilitating transmission) or "ganglionic" (increasing the excitability of anterior horn cells), or both. The initial inhibitory action of eserine, the central inhibitory action of prostigmine and of the "meta" compound may with some confidence be attributed partly, if not wholly, to an anti-cholinesterase action on the central nervous system.

13. The evidence now available indicates the possibility that acetylcholine or a related substance may normally be intimately related to the process of central transmission in somatic reflexes. It would be premature to conclude, however, that the central action of (hypothetically) naturally released acetylcholine would necessarily be identical with its main action on intravenous injection—namely central inhibition.

It is our pleasant duty to express our warm thanks to Sir Henry Dale for his valuable suggestions and critical advice. We are indebted to Dr Stedman for supplies of the "meta" compound and for much helpful chemical information.

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THE INFLUENCE OF TEMPERATURE ON THE ISOLATED KIDNEY OF THE DOG

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One of the cardinal links in the chain of circumstantial evidence supporting the filtration hypothesis in the mammalian kidney has been the fact that a fluid approaching the composition of an ultra-filtrate of plasma emerges from the ureter when the secretory activity has been impaired by asphyxia [Barcroft & Straub, 1910; Marshall & Crane, 1923; Starling & Verney, 1925] or by cyanide [Starling & Verney, 1925; Bayliss & Lundsgaard, 1932; Lundsgaard, 1935]. That the experiments with cyanide prove that urine is formed mainly by a process of filtration in the poisoned kidney, there can be no question. The reversibility of the effects of cyanide encouraged Starling & Verney to "believe we are justified in regarding filtration as playing an important role in the formation of the normal urine of this [heart-lung-kidney] preparation". Even this cautious statement is, however, open to doubt, since it is now known that poisons such as cyanide may not only abolish the secretory activity of a membrane but also render the membrane permeable to substances to which it is impermeable in the absence of cyanide [Richards, 1929]. It is arguable, therefore, that the filtration demonstrated in the cyanided kidney has been produced by the cyanide and does not represent a mechanism present in the unpoisoned kidney.

To allay this doubt about Starling & Verney's interpretation, it would be necessary to show that a similar effect on the kidney is produced by a procedure which, while abolishing the secretory activity as effectively as does cyanide, does not increase the permeability of membranes. Cooling the kidney is, we believe, one such procedure; the general effects of cooling in increasing the urine flow and changing its composition almost to that of a transudate of serum were described by Bickford & Winton [1933] and are illustrated in Fig. 1. Cooling within the range of temperature

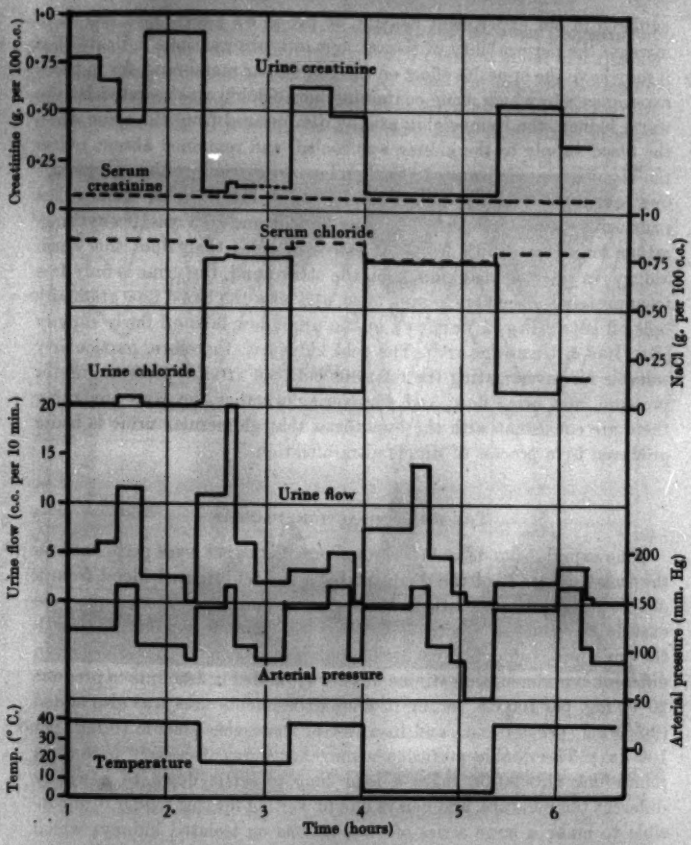

Fig. 1. The effects of changes of temperature on the urine flow, and the chloride and creatinine concentrations in the urine (serum concentrations also shown), and the influence of arterial pressure on these variables at the different temperatures; showing (1) that cooling changes the concentrations of these substances in the urine practically to those in the serum, (2) that this effect is reversible, (3) that if a given change in urine flow is produced by cooling, the change in composition is greater than if produced by rise in arterial pressure. The experiment was performed on an isolated kidney (dog) perfused with defibrinated blood alternately from two pump-lung circulations, the blood in which had been mixed till 1.45.

explored in our experiment has not, so far as we know, been shown to increase the permeability of tissues; our own observations indicate that it may have the opposite effect on the glomerular membrane, for in those experiments in which urine containing hæmoglobin was secreted by the warm kidney, the hæmoglobin usually disappeared from the urine when the blood supply to the kidney was cooled, and remained absent unless the blood was again warmed. Cooling has, moreover, another advantage over cyanide as an experimental procedure, for the cold kidney has relatively constant properties for long periods and survives, if anything, rather longer and with less progressive changes than does the warm kidney; in the cyanided kidney, on the other hand, the urine is only free from protein for the first 10 min. or so, and later the blood flow gradually falls off [Starling & Verney], or the urine flow falls off fairly rapidly [Bayliss & Lundsgaard]. The cold kidney is, therefore, particularly suitable for investigating the relations between arterial pressure, ureter pressure, and urine flow, and discovering whether the laws governing them are consistent with the hypothesis that glomerular urine is being produced by a process of simple ultra-filtration.

The diuretic action of cold

The experiments described throughout this paper were performed on the isolated kidney of the dog perfused with defibrinated blood from a double pump-lung circulation. The apparatus and general technique were exactly the same as described by Whittaker & Winton [1933] with the omission of chloral from the blood; additions to the blood varied in different experiments, creatinine was always added in amounts to produce 20–90 mg. per 100 c.c. serum, in some experiments urea was also added (0·2–0·4 g. per 100 c.c.) and in a few of these also glucose (0·1 g. per 100 c.c.). The double perfusion arrangement was necessary because a pump-lung circulation takes a long time to settle down to a widely different temperature, and delays due to waiting for this render it impossible to make a large series of observations on isolated kidneys which survive in good condition only for a few hours. This difficulty can be overcome by alternating between two separate circulations. That comparable urine flows are obtained with two circulations if proper precautions are taken was shown by Canny *et al.* [1930] on the double heart-lung double kidney preparation.

The diuretic action of cold, shown qualitatively in Fig. 1, is graded according to the degree of cooling as shown in Fig. 2. The urine flow is

only slightly increased by moderate cooling, and becomes progressively more so as the temperature becomes lower. In several of our experiments in which temperatures as low as 3–5° C. were reached, the maximum urine flow seemed already to be reached at or below about 10° C. (Fig. 10). In this respect the effects on the urine flow differ from those of other

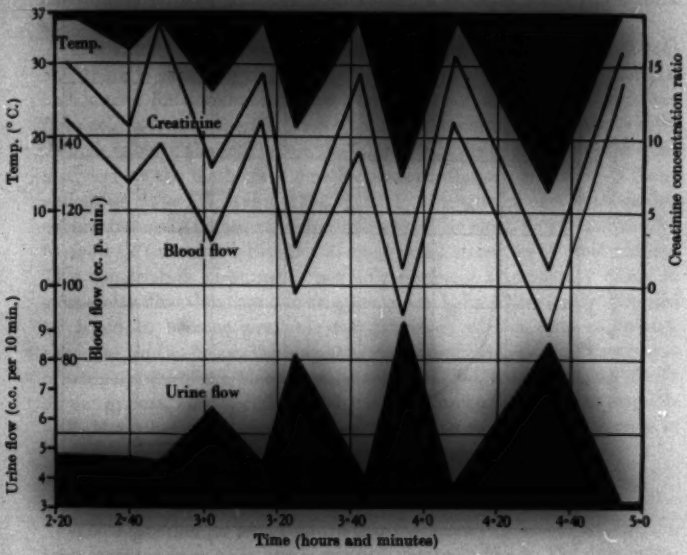

Fig. 2. The graded effects of temperature on the creatinine concentration ratio, the blood flow, and the urine flow of an isolated kidney, showing the general design of an experiment in which each value was determined with reference to observations at a standard temperature (36° C.) immediately before and after that at the lower temperature. B.P. 130 mm. throughout.

properties of the kidney, the creatinine concentration ratio and the blood flow being rather more sensitive to moderate cooling as shown in Fig. 2. This is even more strikingly true of the chloride dilution ratio, which, as will be shown, already reaches its minimal value at about 18° C. The variation in urine flow within a range of temperature within about 5° of 8° C. is, however, small, and it will be convenient to refer to the change of flow due to a fall of temperature from about 36° C. to within this range

as the cold diuresis, and to the kidney within this range as the "cold kidney". This diuresis is very different in different kidneys and under

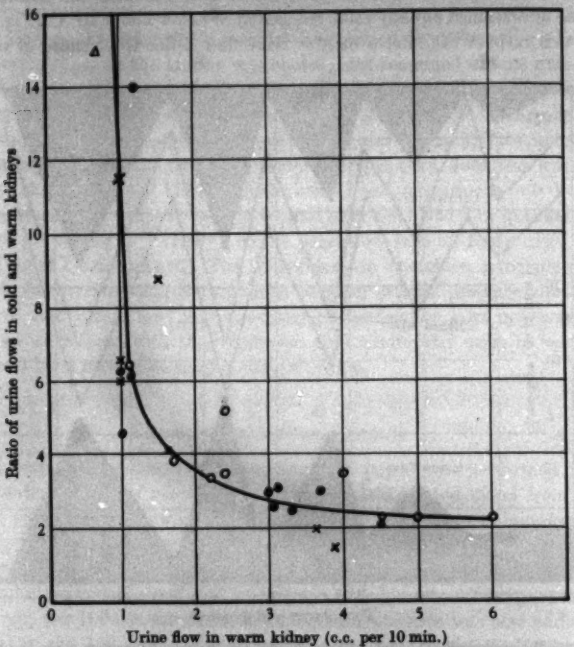

Fig. 3. The relation between the urine flow in the warm (36–38° C.) kidney and the diuretic action of cooling to 3–13° C., expressed as the ratio of the flow in the cold kidney to that in the warm kidney. The variation in urine flow in the warm kidney was due to variation in the arterial pressure or the urea content of the blood, or to fortuitous circumstances. That arterial pressure itself plays no important part is shown by the facts that: triangles represent pressures of 90 mm., dots 100–130 mm., crosses 140 mm., and circles 160–200 mm. Hg. Compiled from observations on seventeen kidneys. As the urine flow in the warm kidney approaches zero, the ratio depicted approaches infinity.

different conditions; it ranges commonly from about two- to ten-fold. One factor stands out as related to the magnitude of the cold diuresis, namely, the absolute magnitude of the urine flow in the warm kidney.

The greater the initial urine flow, the smaller the cold diuresis, as shown in Fig. 3. It is a curious circumstance that it does not appear to make much difference to this relation whether the initial urine flow is high due to a high arterial pressure (as shown in the figure), to urea in the blood, or to the unknown agents which frequently operate to produce varying flows from isolated kidneys from different animals under apparently similar conditions.

If a kidney be suddenly switched from a warm circulation to a cold one, there is usually a transient fall of urine flow preceding the diuresis, and lasting for a minute or two. Conversely if the organ be switched from cold to warm, there is often a transient rise of urine flow above even its high value in the cold before the fall sets in. This phenomenon has been analysed by the methods of simultaneous continuous recording of blood and urine flows [Winton, 1936 *b*], from which it appears that sudden cooling induces a transient vaso-constriction, the blood flow falling maybe to one-half the value which it will reach when steady in the cold kidney. This constriction presumably not only produces a temporary check in glomerular filtration, but allows space in the kidney previously occupied by blood to become available for distension of the urine-containing elements, and so delays the output of urine from the ureter. If the change of temperature of the blood is sufficiently gradual, these transient effects do not appear, corresponding with the well-known fact that the arteriole wall, like other plain muscle, is more sensitive to the rate of change of temperature than to the magnitude of the change itself.

The effect of cooling on the renal blood flow

The reduction in blood flow when the kidney is cooled to varying temperatures from body temperature is shown in Fig. 4. The dots represent observations on ten kidneys. The continuous line represents the changes in flow which would be obtained in a glass viscometer; it is constructed from the data of Hosking [1909] on the variation of the viscosity of water with temperature, and on a factor for the ratio of the viscosity of blood (similar to that used in our experiments) to the viscosity of water at different temperatures which Dr L. E. Bayliss has derived from his unpublished experiments and has kindly allowed us to use. Taking into consideration that different experiments were performed with bloods of varying corpuscular concentration and at different arterial pressures, and that some readings of blood flow were taken before the transient vaso-constriction due to sudden cooling had completely passed off, we may infer that the resistance of the vascular system of the kidney

to blood flow undergoes no considerable change due to cooling other than that due to change in the viscosity of the blood. If anything, the change is in the direction of cooling producing a smaller increase in resistance in

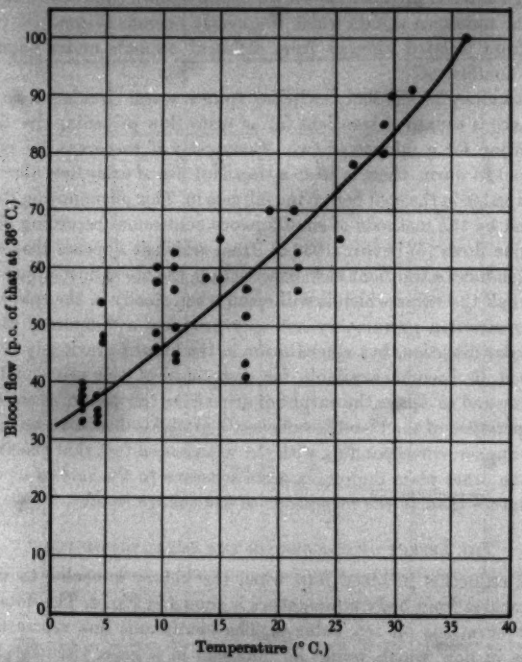

Fig. 4. The effect of temperature on the blood flows through isolated kidneys (at various arterial pressures); flows are expressed as a percentage of the flow through the same kidney at the same pressure at 36° C. Dots represent measurements on kidneys. The line represents the change of flow with temperature of similar blood flowing in a glass viscometer [constructed from data by Hosking, 1909, and Bayliss, 1936].

the kidney than in the viscometer, as illustrated by the following experiment. A kidney and the glass viscometer specified by Whittaker & Winton [1933] were arranged in perfusion parallel and a pressure of 142 mm. Hg applied. The figures are for blood flows expressed as a percentage of those when the circulation was warm.

Temp. °C.	Viscometer	Kidney	Temp. °C.	Viscometer	Kidney
38	100	100·0	5	42	47
6	41	45·5	35	100	100

One factor which can be shown to affect the reduction in blood flow due to a given degree of cooling is the arterial pressure; this is seen in an experiment designed like that of Fig. 1 if the blood flow in a cool kidney is expressed as a percentage of that in the kidney at 36° C. at the same pressure, and such percentages plotted against the arterial pressure as

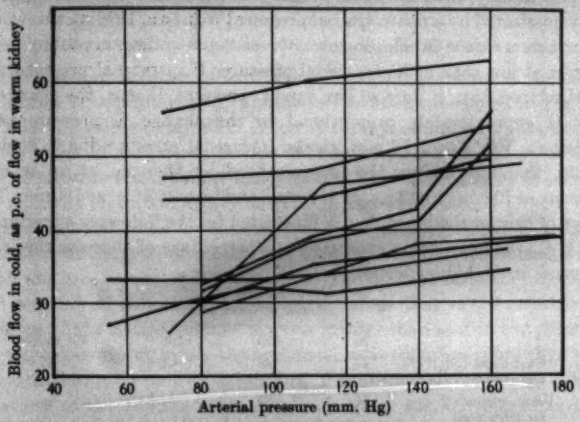

Fig. 5. The effect of arterial pressure on the blood flow through the cold kidney expressed as a percentage of the flow through the warm kidney at the same pressure. The curves show that cooling makes the blood flow fall proportionately to a lower value at low than at high arterial pressures. (Each curve represents a single kidney at a given temperature.)

in Fig. 5. The figure includes all the observations we have made on this point; each of the curves shows that a given fall in temperature produces a greater fall in blood flow at a lower than at a higher arterial pressure. Such increase in resistance to blood flow may be due to vaso-constriction, to increased viscosity, or to increase in intrarenal pressure exerting a back pressure at the venous end of the circulation. Of these, vaso-constriction is unlikely because the fall in blood flow for a given change in temperature is often less than the corresponding fall in flow in a glass viscometer. There is no doubt that the viscosity of blood increases on cooling, and since the apparent viscosity increases at low rates of flow,

and presumably the temperature coefficient of the viscosity will increase along with it, it is just possible that this contributes to the effect of arterial pressure under discussion. If the kidney behaves in this respect like the hindlimb, this consideration can, however, be ignored as quantitatively too small to be significant over the relevant range of arterial pressure [Whittaker & Winton, 1933, Table 3].

The increase in intrarenal pressure due to cooling to about 9° C. may be taken on the average, as about 13 mm. Hg [Winton, 1934 *a*]. If in the cold kidney, as in the warm kidney, the intrarenal pressure is practically unaffected by a rise in arterial pressure [Winton, 1936 *a*], this would constitute a reason for the increase of resistance to flow on cooling being greater at low than at high arterial pressure. If intrarenal pressure acts on the blood flow, it does so like venous pressure, that is, the change of flow is approximately proportional to the change in pressure head [Winton, 1931 *b*]. A 13 mm. rise in intrarenal pressure due to cooling would, therefore, reduce the pressure head by 18·5 p.c. at an arterial pressure of 70 mm., by 12 p.c. at 110 mm., and by only 8 p.c. at 160 mm. The effect of this on the blood flow is illustrated by the following experiment at 11° C., the flows being expressed as a percentage of those at the same pressure in the kidney at 36° C.

	Kidney arterial pressure mm.			
	70	110	160	Viscometer
Blood flows (p.c.) observed at 11° C.	44	45	49·5	47
Flows corrected for intrarenal pressure change	52	51	53·5	—

This correction for increase in intrarenal pressure yields values of the flows at different pressures which are within the range of experimental uncertainty. Fig. 5 indicates, however, that the effect of arterial pressure is often rather greater than in this experiment, and it seems not unlikely that some factor additional to the change in intrarenal pressure may contribute to the effect.

If the increased obstruction due to the rise in intrarenal pressure on cooling be taken into account together with the fact that the observed reduction in blood flow in the kidney is, if anything, rather less than that in the viscometer for a given fall in temperature, it would appear that some other factor must be affecting the blood flow, which lessens the resistance to flow of the kidney in the cold as compared with warm. This may represent vaso-dilation, or it may be due to the reduction in the rate

of glomerular filtration due to cooling (cf. Figs. 9, 10) which would lead one to expect such a reduction in resistance to blood flow [Winton, 1932], and would also contribute to the effect of arterial pressure named above.

Heidenhain's [1883] contention that the urine flow is determined by blood flow rather than by glomerular pressure has been refuted as far as observations on the warm isolated kidney are concerned [Winton, 1931 *b*], but there is perhaps no clearer demonstration of the dissociation of blood flow and urine flow than that provided by change of temperature as in Fig. 2.

The effect of cooling on the compositon of the urine

The essential features of the changes with temperature of the concentrations of creatinine and chloride in the urine are shown in Fig. 6. To make the values obtained in different kidneys comparable, the urine-plasma ratios of the concentrations of creatinine for each kidney at one arterial pressure but different temperatures have been rescaled in such a way that the mean concentration ratio in the warm kidney (36–37° C.) is scored as 100 while a concentration ratio of unity remains 1. The average of the thirty determinations of the concentration ratio of creatinine which are here scored as 100 was 15·3.[1] The dilution ratios of chlorides were rescaled on an exactly similar plan, except that as the chloride in the urine of the isolated kidney is never greater than that in the plasma, it is more convenient to use the reciprocal ratio (dilution ratio) than the concentration ratio as with creatinine. The average of the thirty determinations of the dilution ratio of chloride which are here scored as 100 was 7·7, i.e. the plasma chloride was 7·7-fold that in the urine. The general design of the experiments from which the data were obtained is indicated by Fig. 2, and a value corresponding with a low temperature could therefore be expressed with reference to the mean of the values corresponding to body temperature (36–37° C.) determined immediately before and after.

One of the most striking consequences of arranging the results in this way is that it is immediately apparent from Fig. 6 that the mechanism controlling the dilution of chloride is more sensitive to change in temperature than that controlling the concentration of creatinine. For a 10° C. fall below body temperature the reduction in the chloride dilution ratio is 81 p.c., whereas that in the creatinine concentration ratio is only 56 p.c. The second point illustrated by the curves in Fig. 6 is that, whereas

[1] Rather high urine flows were included in these experiments in order that a large series of observations could be made during the limited period of survival of a kidney.

the chloride dilution ratio falls to unity on cooling to or below about 18° C., the concentration ratio of creatinine only reaches its lower limit at or below about 12° C., and then remains substantially above unity. Forty-five determinations of the chloride dilution ratio in ten cold

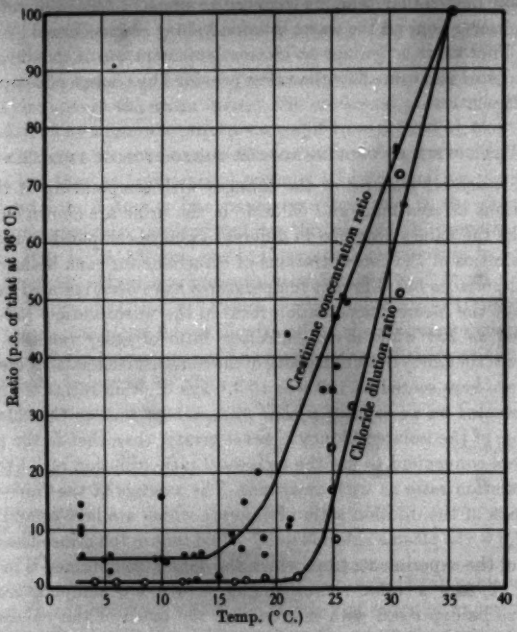

Fig. 6. The influence of temperature on the concentration ratio of creatinine (dots) and on the dilution ratio of chloride (circles). Both ratios are rescaled so that the ratio in the warm kidney becomes 100 while a ratio of unity remains so.

kidneys gave a mean of $1{\cdot}00 \pm 0{\cdot}03$; ninety-six determinations of the creatinine concentration ratio in twenty-one cold kidneys gave a mean of $1{\cdot}5 \pm 0{\cdot}25$.[1] Both these results agree well with the consequences of cyanide poisoning of the isolated kidney [Bayliss & Lundsgaard, 1932]. Lundsgaard [1935] reports the slightly higher concentration ratio of creatinine of 1·79 in the presence of $M/1000$ cyanide as the mean

[1] It is likely that this mean is a little too high, as it is based in part on observations made rather soon after cooling. Possibly 1·3 would be a fairer value.

of four experiments, which gave values ranging from 1·3 to 2·3. Bayliss & Lundsgaard's earlier experiments had, however, shown that a concentration of cyanide of *M*/500 may produce a fall of the ratio to 1·5 after it had been 2·3 in the presence of only *M*/1450; and this suggests that the explanation of the discrepancy between the ratio in the cold kidney and in Lundsgaard's cyanided kidney is due to incomplete poisoning in the latter.

The abolition of secretory activity is what might be expected in the cold or cyanided kidney, and the behaviour of chloride is in accordance with such expectation. The survival of the power of concentrating creatinine, and presumably [Bayliss & Lundsgaard] of glucose and phosphates is not easy of explanation. Bayliss & Lundsgaard suggested that cyanide impaired but did not destroy secretory activity. We have, however, not found that further cooling below about 12° C. reduces the concentration ratio of creatinine any further. This is best seen in single experiments, but sufficiently indicated by Figs. 2 and 6. Time plays some part in the action of cooling, the full effect may not develop till 15–20 min. after the change of temperature, although at the high urine flow which results the uncertainty due to the dead space in the kidney occupies only a minute or two. After this period, there is, however, no progressive fall in the concentration ratio, and there may even be a rise. In one kidney kept at 5° C., 25 min. after lowering the temperature the urine flow was 13·3 c.c. per 10 min., and the concentration ratio of creatinine 1·46; 1 hour later at the same temperature and pressure the urine flow was 11·6 c.c. per 10 min. and the concentration ratio 1·59. This is like the increase of concentration ratio reported by Bayliss & Lundsgaard as occurring during the spontaneous falling off in urine flow which occurs after the cyanide diuresis has reached its height. We found the effect in some but not all our observations on this point. Apart from this effect, Bayliss & Lundsgaard report an experiment on a cyanided kidney in which doubling the urine flow by a 20 mm. rise in arterial pressure reduced the concentration ratio from 1·48 to 1·45, subsequent fall by 40 mm. increased the ratio to 1·95. They suggest that this residual concentration ratio is governed by the law that increased urine flow is accompanied by a lower ratio as in the unpoisoned kidney.

The residual creatinine concentration ratio in the cold kidney is plotted against arterial pressure in Fig. 7. The results obtained from all eleven kidneys examined for this relation are depicted, and some of the more discordant slopes derive from earlier experiments in which the importance of allowing sufficient time after cooling for equilibrium to be

achieved was not fully appreciated. It is, we believe, fair to infer from our results that a variation of arterial pressure over a wide range, producing changes in urine flow varying in different experiments from three- to twenty-fold, produces no substantial change in the residual creatinine concentration ratio. How different the cold kidney is in this respect from the warm kidney is shown by comparing Figs. 7 and 8. In Fig. 8 the creatinine concentration ratio is again plotted against arterial pressure

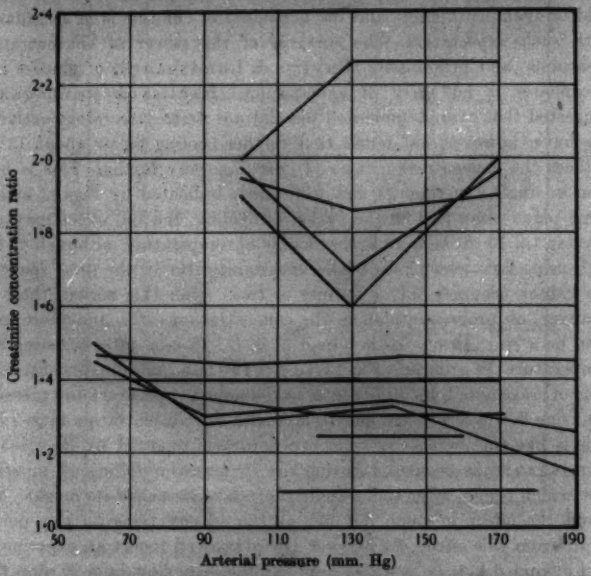

Fig. 7. The relation between arterial pressure and the creatinine concentration ratio in eleven cold kidneys. Each curve represents a different kidney, at temperatures varying from 3–13° C. To show that increase in arterial pressure does not systematically reduce the concentration ratio in the cold, as it does in the warm kidney (cf. Fig. 8).

in an unselected series of ten kidneys, and the systematic relation between arterial pressure and concentration ratio is unequivocal. The two lowest curves, and that depicted by squares, differ somewhat from the rest because they were obtained from kidneys under the influence of fairly high concentration of urea or sodium sulphate. The dilution ratios of chloride

in the warm kidney are likewise dependent on arterial pressure, but the ratio in the cold kidney appears to be independent of arterial pressure or

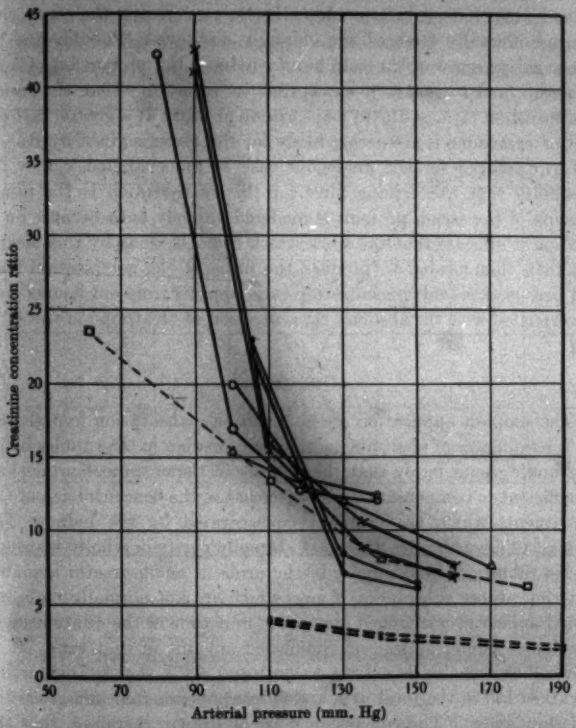

Fig. 8. The relation between arterial pressure and the creatinine concentration ratio in ten kidneys at 36–38° C., showing that the ratio is systematically reduced by increase in pressure, in contrast to the cold kidney (Fig. 7). Note that the lower two curves and that denoted by squares represent kidneys under the influence of a considerable concentration of urea.

urine flow. This fact is in accordance with theoretical expectation, and it has therefore not been considered sufficiently important to give the detailed evidence in a figure.

If, as seems likely, we may attribute to the residual creatinine concentration ratio in the cold kidney the properties (*a*) of being unaffected by further cooling, and (*b*) of being independent of arterial pressure and concomitant changes in urine flow, the laws governing the composition of urine normally secreted are glaringly disobeyed. Possibly the high intrarenal pressure in the cold kidney reduces the proportion of active glomeruli, and so makes it susceptible to variation. That the residual concentration ratio is due to reabsorption of water at a greater rate than that of creatinine is altogether likely for the reasons given by Bayliss & Lundsgaard for the analogous case of the cyanided kidney. The possibility that the driving force for this reabsorption is the osmotic pressure of the serum proteins is rendered unlikely both because on calculation it appears that the work which could be done by this means is less than that needed to produce the urine of the composition found, and because residual concentration ratio appears from our figures not to be correlated with the absolute concentration of creatinine in the serum.

Discussion

The simplest application of the filtration-reabsorption hypothesis to the consequences of abolishing active reabsorption in the tubules by cold or cyanide would imply that the increase in the output of urine should be sufficient to compensate for the decrease in the concentration of those constituents which are normally concentrated by the kidney. Thus, substances like chlorides which are normally partly or wholly reabsorbed by the tubules, should appear in the urine in much greater amount in unit time; those substances, if any, which are not normally reabsorbed should appear in unchanged amount in unit time in the dilute urine; no substances should appear in diminished amount in unit time. It was shown, however, by Starling & Verney [1925], that cyanide induces a marked fall in the total urea and sulphate eliminated in a given time, and they state: "The conclusion must be drawn, therefore, that these bodies are actively secreted into the glomerular filtrate by the tubule cells during life." Alternative explanations of this fall have been put forward, based on the possibility that either less glomerular fluid is formed, or that part of that formed leaks back into the blood across a tubular membrane rendered abnormally permeable by cyanide. Bayliss & Lundsgaard [1932] adopt the latter explanation for the fall in the rate of creatinine excretion which they observed in poisoning the kidney with cyanide, and support this interpretation by reference to Richard's

[1929] experiments on frog's kidneys in which he demonstrated such abnormal permeability of the tubules in the presence of cyanide and mercuric chloride.

Our observations on cooling are closely parallel to those mentioned as due to cyanide. One may summarize the results roughly by saying that at normal rates of urine flow (0·2 c.c. per min.) the concentration ratio

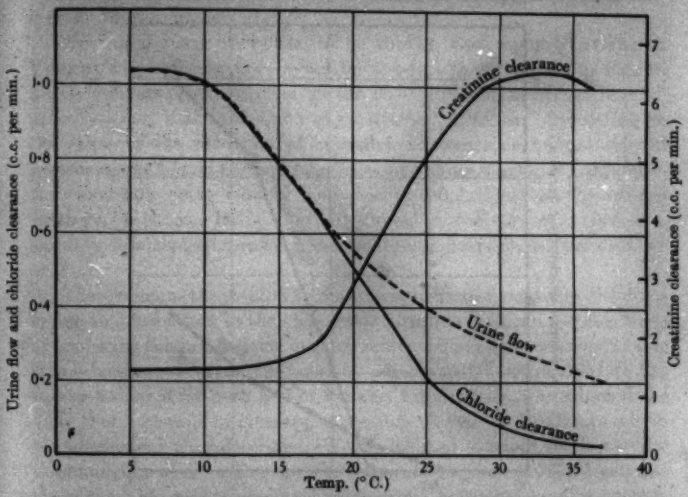

Fig. 9. The effect of temperature on the urine flow, the chloride clearance, and the creatinine clearance in the isolated kidney. The data from which these curves are constructed are derived from observations on twelve kidneys and are averaged so as to produce a representative summary of the results. The small increase in creatinine clearance on slight cooling is not necessarily significant.

of creatinine is reduced by cooling, about twentyfold, whereas the urine flow is increased only fivefold. The product of these two values, the creatinine plasma-clearance, is thus reduced about four-fold (the total creatinine excreted in unit time is proportional to the clearance if the serum creatinine remains constant).

The graded effects of temperature on the creatinine clearance, the chloride clearance similarly calculated, and on the urine flow have been observed on twelve kidneys, and these observations are summarized in

the composite curves shown in Fig. 9. Bayliss & Lundsgaard's explanation of the reduction in the creatinine clearance is not so convincingly applicable to the effects of cooling as of cyanide for the following reasons: (*a*) cooling within the range here discussed has not been shown to render membranes abnormally permeable; (*b*) such indications of changes of permeability as a result of cooling as we have had in our experiments

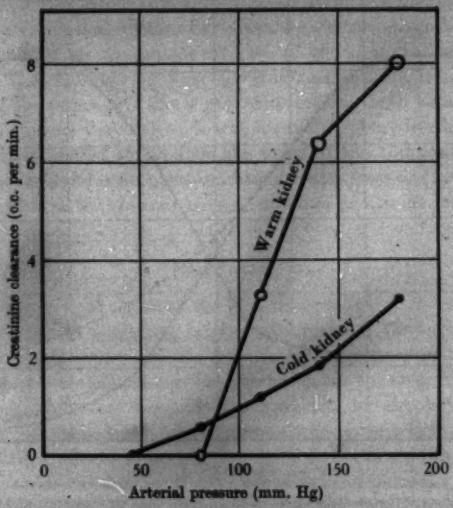

Fig. 10. The relation between arterial pressure and the creatinine clearance in an isolated kidney at 37° C. and at 10° C.

were in the opposite sense, for in those experiments where the urine from the warm kidney contained hæmoglobin, the urine of the cold kidney was usually free from this constituent. It is, however, arguable that impermeability to substances such as urea and creatinine is such an unusual feature in other tissues that this property of the renal tubules must be due to specific vital activity which is abolished on cooling. But this hypothesis is difficult to reconcile with the evidence obtainable at lower arterial pressures, Fig. 10 representing a typical experiment. It will be seen that whereas the creatinine clearance is reduced by cooling at normal arterial

pressures, the reduction becomes less as the arterial pressure approaches the value at which urine flow ceases in the warm kidney, and below this the effect of cold is actually to increase the clearance. Abnormal permeability resulting in a leak from the tubules should, one might expect, raise the minimum arterial pressure compatible with urine flow; in fact, the least effective arterial pressure in the cold kidney is usually about 40–50 mm. Hg, whereas in the warm kidney in the absence of diuretics it is 20–30 mm. higher.

We would seem, therefore, to be thrown back upon Starling & Verney's hypothesis, were it not for the fact that the data in Fig. 10 are as difficult of explanation on the basis of abolition of "secretory" activity. For, if the reduction of creatinine "secretion" by cooling be the cause of the reduction of creatinine clearance at normal arterial pressures, one should find a similar reduction at lower arterial pressures—the blood flow being reduced much less in proportion than the arterial pressure [Winton, 1932]. Fig. 10 shows, on the contrary, that at a suitably low arterial pressure the creatinine clearance is increased by cooling.

There remains the possibility that the reduction of creatinine clearance is due to a reduction in the glomerular filtration rate. Of possible ways of producing this, a reduction in glomerular capillary pressure, comparable in mechanism with that produced by adrenaline [Winton, 1931 *d*], has been considered by both sets of workers with cyanide who adopted the view that a change in glomerular pressure played no significant role in the phenomena they described. We are inclined to adopt the same view in connexion with cooling for the following reasons: (*a*) the creatinine clearance is reduced to about the same extent by cyanide and by cooling, but cyanide increases and cooling decreases the blood flow, (*b*) the increase in clearance due to cooling at low arterial pressures seems incompatible with the supposition that the reduction in clearance at higher pressures is due to diminution in glomerular pressure, (*c*) the maximum ureter pressure is raised from an average of 30 mm. in the warm isolated kidney [Winton, 1931 *a*] to 70 mm. in the cold kidney (arterial pressure 128 mm. Hg)—this should not be taken as necessary evidence of a rise in glomerular pressure, as a similar rise in maximum ureter pressure accompanies urea diuresis [Winton, 1934 *b*], (*d*) an experiment on the effects of cooling on the urine flows of a pair of kidneys, one with raised venous pressure and the other with raised ureter pressure so adjusted that the urine flows remained equal, indicated no great change of glomerular pressure [Winton, 1931 *c*].

Arterial pressure 120 mm. Hg

Temp. °C.	Left kidney: Venous pressure mm. Hg	Left kidney: Urine flow c.c./10 min.	Right kidney: Ureter pressure mm. Hg	Right kidney: Urine flow c.c./10 min.	Glomerular pressure mm. Hg
36·0	17·5	0·75	9·5	0·72	65·0
11·0	17·5	3·60	9·5	4·20	—
9·2	58·0	1·20	32·0	1·20	66·0
9·2	0	14·40	0	14·40	—

The estimation of a change in glomerular pressure on cooling is complicated by the development of an intrarenal pressure which is often almost as high as, or may be higher than the maximum ureter pressure in the warm kidney; the set of figures for 11° C. is probably invalidated on this account, and because cooling was still in progress. The small rise of glomerular pressure shown is well within the range of experimental uncertainty, but the method of calculating it may not be valid, as previously indicated [Winton, 1931*c*].

If we assume that cooling produces no considerable change in glomerular capillary pressure, we are left with various possibilities of a reduced filtration rate, including that due (1) to increased intrarenal pressure, (2) to reduced permeability of the glomerular membrane, and (3) to increased mean osmotic pressure of the plasma proteins in the glomerular capillaries under conditions of reduced blood flow. Neither of the latter agents is likely to be a significant factor because cyanide produces much the same effects as cooling: the permeability of the glomerular membrane which may be reduced by cooling, as indicated by the observations on hæmoglobinuria mentioned above, is probably increased by cyanide which in sufficient concentration or acting for a sufficient time produces albuminuria; increased osmotic resistance to filtration due to the reduction of the blood flow to about one-half on cooling must be quantitatively unimportant in reducing the filtration, since about the same reduction in filtration is produced by cyanide which, according to Bayliss & Lundsgaard, is accompanied by about a 30 p.c. increase in blood flow, and since the reduction in creatinine clearance which may provisionally be adopted as a measure of the glomerular filtration rate [Rehberg, 1926; Shannon, 1935] is about fourfold while the reduction in blood flow is only about twofold.

That a rise in intrarenal pressure results from cooling is evidenced qualitatively by the great increase in volume and tenseness of the kidney pointed out in our previous communication [1933]. An attempt to measure this rise in intrarenal pressure in a series of kidneys was reported by one of us [1934*a*]. With the reserve necessitated by doubt as to whether the method of measurement based on ureter pressure-urine flow curves is valid, though this method gives results similar to that of ex-

posing the kidney to pressures below atmospheric [Herman & Winton, 1936], and the further reserve due to great individual variation in different kidneys, we may attempt to assess how great a part in the reduction of filtration may be attributed to this rise in intrarenal pressure. From the figures given [1934 *a*] it can be calculated that considerably less than one-half the reduction in creatinine clearance on cooling can be attributed to the rise in intrarenal pressure. The remainder of the reduction remains unexplained. It is possible that the latter is due to reduction in glomerular filtration which may accompany such drastic inhibition of reabsorption of water; for the pressure necessary to drive water down the tubules distal to the site of reabsorption presumably increases at about the same rate as the increase in urine flow, i.e. four or fivefold, and is also about doubled by the increased viscosity. This pressure increase is likely to be quantitatively more important when the pressure before cooling is already high, and this conception would, therefore, readily explain the result shown in Fig. 3 that the degree of increase in urine flow due to cooling is intimately connected with the initial urine flow. The possibility of a reduction in the proportion of active glomeruli, and a consequent reduction in clearances due to cooling, would be favoured by the apparent constancy of the residual creatinine concentration ratio.

In conclusion we may consider the two facts: (1) that cooling reduces the minimum arterial pressure at which urine is secreted by 20–30 mm., and (2) that cooling increases the maximum ureter pressure at constant arterial pressure by 30–40 mm. Hg. In both respects the cold kidney behaves as it should on the filtration-reabsorption hypothesis, the warm kidney (in the absence of diuretics) does not. Similar effects in reducing the minimum effective arterial pressure and in raising the maximum ureter pressure are produced by tubule poisons, and by diuretics such as urea and sulphate [Winton, 1934 *b*]. These facts are exceedingly difficult to reconcile with either the filtration-reabsorption hypothesis or the secretion hypothesis. The simplest explanation would be to suppose that a leak from the distal tubules was a normal feature in the warm kidney, urine leaking back into the blood, and that this leak was reduced by cooling and by the other agents named. But if this were true, it would increase the magnitude of the reduction in creatinine clearance which must be attributed to some of the same agents at relatively high arterial pressures, and would imply that these agents produce this effect through change in a second variable, which cannot be identified with certainty until further information is available.

Summary

1. Cooling the blood perfusing the isolated kidney of the dog from body temperature to anywhere within the range 3–13° C. changes the composition of the urine substantially to that of a serum transudate. The kidney survives at least as long in the cold as in the warm, and recovers completely on rewarming (Figs. 1, 2).

2. On cooling, the urine flow increases, reaching a maximum at about 10° C. The maximum increase may vary from about two to tenfold or more according as the initial urine flow is high or low (Fig. 3). At a normal flow (2 c.c. per 10 min.) the increase is of the order of fivefold. A fall of 10° C. below body temperature produces about 15 p.c. of the maximum change produced by cooling in the urine flow (Fig. 9).

3. The minimum arterial pressure which will produce urine in the warm kidney uninfluenced by diuretics is usually 70–80 mm. Hg. In the cold kidney it is usually 40–50 mm. Hg, which accords more satisfactorily with the implications of the filtration hypothesis (Fig. 10).

4. Cooling reduces the blood flow in approximately the same proportion in the isolated kidney and in a glass viscometer (Fig. 4). If the increased obstruction to the blood flow due to the rise in intrarenal pressure on cooling be taken into account, one can infer a factor in the resistance of the kidney to the flow which decreases on cooling. The reduction in blood flow on cooling a kidney is proportionately greater at low than at high arterial pressures (Fig. 5).

5. The chloride concentration in the urine of the isolated kidney, always low at body temperature, is increased by cooling so that, below about 18° C., it becomes indistinguishable from the chloride concentration in the serum, and remains so however the urine flow be varied by changing the arterial pressure. A fall of 10° C. below body temperature produces about 80 p.c. of the maximum change produced by cooling in the chloride concentration (Fig. 6).

6. The creatinine concentration in the urine can be reduced by cooling (as by cyanide) only to about one and a half times that in the serum; it reaches this minimum value by about 12° C. A fall of 10° C. below body temperature produces about 50 p.c. of the maximum change produced by cooling in the creatinine concentration (Fig. 6).

7. No change in the residual creatinine concentration ratio of 1·5 in the cold kidney could be detected when the urine flow was varied by changing the arterial pressure (Fig. 7), though the ratio in the warm kidney varies considerably with arterial pressure (Fig. 8).

8. If equal changes in urine flow be produced either by change in arterial pressure or by change in temperature, the change in composition of the urine, though qualitatively in the same direction, is quantitatively much greater with the latter than with the former agent (Fig. 1).

9. The creatinine clearance (proportional to the total creatinine output per min. in the urine at constant serum concentration) is reduced by cooling at normal arterial pressures to about one-quarter (Fig. 9). The reduction is only in part due to the rise in intrarenal pressure. It is comparable with that produced by cyanide, but it cannot be explained simply by supposing that three-quarters of the creatinine is normally "secreted" into the lumen, nor simply by supposing that the reduction is due to increased reabsorption from the tubules, because the reduction disappears if the arterial pressure be lowered to about 80 mm.; at still lower pressures there is, instead, an increase in the creatinine output on cooling (Fig. 10).

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ACTION POTENTIALS OF NORMAL MAMMALIAN MUSCLE. EFFECTS OF ACETYLCHOLINE AND ESERINE

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In a recent paper Brown *et al.* [1936] have shown that injection directly into the arteries of a normal mammalian muscle of small amounts of acetylcholine causes a quick twitch-like contraction only little different in time relations from the contraction evoked by a single volley in the motor nerve. Theoretical considerations led to the deduction that the acetylcholine contraction was probably a short asynchronous tetanus of the muscle fibres and in a footnote (p. 420) reference was made to the confirmation of this by electrical records. In the present paper an account is given of the action potentials associated with the excitation of muscle by injected acetylcholine.

Brown *et al.* also showed that a small dose of eserine potentiated the response of muscle to single maximal volleys in the motor nerve, changing the muscular contraction from a single twitch into a brief tetanus. This phenomenon has subsequently been shown by Bacq & Brown [1937] to be a property common to other substances antagonizing choline esterase. In this paper the mechanical and electrical response of the muscle under eserine has been examined in detail, in relation especially to the frequency at which the poisoned muscle contracts repetitively in response to a single nerve volley. A number of experiments have been done in which the muscle has been subjected to two volleys in the nerve at varying time intervals. This has been considered in the light of the known failure of the eserine-treated muscle to respond to stimuli delivered above a very low frequency [Briscoe, 1936; Bacq & Brown, 1937] and the depressant effects of acetylcholine injected after eserine. Some

preliminary observations have also been made on the effects on the response of muscle to nerve volleys, of exposure to supraliminal and subliminal concentrations of acetylcholine.

Methods

Cats have been used throughout this investigation, either spinal or decerebrate or anæsthetized with chloralose, a substance which Bacq & Brown found to be without significant effect on the reactions of muscle

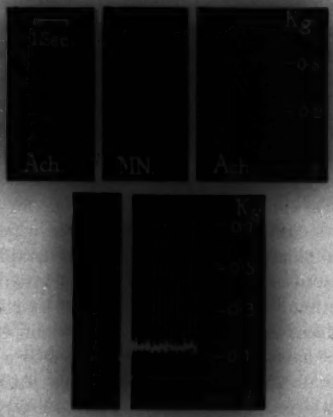

Fig. 1. Cat. 2·6 kg. Decerebrated. Record of contractions of soleus. (*a*) Close arterial injection of 2·5γ ACh., maximal motor nerve twitch and injection of 5γ ACh. respectively. (*b*) Effect of intravenous administration of 0·8 mg. eserine on response of muscle to maximal nerve volleys at 10 sec. intervals.

to eserine and acetylcholine. Most of the experiments on acetylcholine injection have been made on the gastrocnemius muscle with natural circulation by the method of Brown *et al.* [1936], injecting through the tibial artery, but recently it has been found possible to use the soleus. This muscle, consisting of "red", slowly contracting fibres, responds just as well to injected acetylcholine as does the gastrocnemius. The difference in time relations between the contraction evoked by acetylcholine and the response to a single motor nerve volley is less pronounced than in gastrocnemius on account of the slowness of the nerve twitch (Fig. 1). This figure also shows the very dramatic potentiating effect of eserine

upon the motor nerve twitch of this muscle. The soleus was prepared as follows. The gastrocnemius tendon was severed near the *os calcis*, leaving the soleus still attached to the bone, and reflected backwards to expose the tibial artery just after its passage between the heads of the muscle. The branches of the tibial artery to the soleus and to the knee joint were defined and the latter were ligated together with all branches of the artery to the soleus, except those actually supplying the muscle. The tibial artery was exposed down to the interosseous membrane, it was tied and a needle cannula was inserted into the central end. Injections were made through this cannula, the tibial artery central to the origin of the vessel supplying the soleus being clamped immediately before the injection was made. The gastrocnemius, tibialis anticus and other muscles of the foot were immobilized by section of the branches of the popliteal and tibial nerves and the leg was fixed for recording by drills in the condyles of the femur and the lower end of the tibia and fibula.

In many of the experiments in which the action of eserine was studied, without arterial injections being made, the records were taken from the tibialis anticus. The appropriate drills in the bone for the muscle under experiment were secured to a heavy iron table which carried, on an adjustable vertical pillar, a stout torsion wire myograph of the "frictionless" pattern described by Eccles & Sherrington [1930]. In all experiments the stimuli to the nerve were given by glass-shielded silver wire electrodes, placed on the sciatic nerve a few cm. distal to the origin of the nerves to the hamstrings. The stimuli, timed by a Lucas pendulum, were break induction shocks from coreless coils placed far apart and shielded in metal boxes. The primary current (2 V.) was varied by a series resistance. In most experiments stimuli of a strength 50–100 p.c. greater than that required to set up a maximal volley were used. When two stimuli were employed, at very short time intervals, preliminary records were taken to ensure that the second, falling in the relative refractory period of the nerve, was sufficiently strong to evoke a second response from the muscle.

It was usually found desirable after completion of the preparation to leave the animal with the stimulating electrodes in place for $\frac{1}{4}$ to $\frac{3}{4}$ of an hour, since the strong stimuli used sometimes caused, when the nerve was freshly dissected, the setting up of a few repetitive discharges.

The arrangements for electrical recording were as follows. The metal plate to which the immobilizing drills were attached was insulated from the iron table by fibre discs and the hook connecting tendon to myograph was interrupted by a section of insulating fibre. The leads from the muscle

for the action potential following nerve stimulation were either two silver pins, one in the tendon connected to the input grid and the other, connected to earth, inserted in the belly of the muscle or concentric needle electrodes [Adrian & Bronk, 1929] consisting of a hypodermic needle with a No. 36 gauge enamelled copper wire just appearing at the tip. The needle body was connected to earth and the copper wire to grid. For the action potentials evoked by acetylcholine injection, either the concentric needle electrodes were used or, to obtain closer localization, electrodes consisting of two enamelled No. 47 gauge wires twisted together and passed down a hypodermic needle or a fine glass capillary [Adrian & Bronk]. With the belly-tendon leads and the concentric needle electrodes no electrical screening of the animal was needed, but with the twin wire electrodes it was found necessary to enclose the animal in a large earthed metal box with a lid covering as much of the front as was compatible with manipulating the syringe for injection. It was found possible to effect the arterial injections without introducing artefacts, if the operator earthed himself by grasping a saline pad on the screening box and took care to touch only the glass piston of the syringe at the moment of injecting. The amplifier consisted of four resistance-capacity coupled stages connected to four pentodes in parallel actuating a Matthews' oscillograph. With belly-tendon leads, the last two stages and large coupling condensers, giving a fall to half value in 0·52 sec. with a rectangular impact potential, were found satisfactory. With the concentric electrodes, when recording the responses to nerve stimulation, three stages were used with small coupling condensers to minimize base-line drift. When recording action potentials from acetylcholine injection, the four stages were found to be necessary, but only seldom was more than half the maximum amplification ($10\,\mu$V.$=$5 mm. on camera) required.

Acetylcholine was injected in acid saline (pH 4) in volumes of 0·5 c.c. for gastrocnemius and in 0·25 c.c. for soleus.

Eserine when used was administered intravenously, the circulation being protected by the previous injection of 1 mg. atropine.

Results

Brown *et al.* [1936] dealt in considerable detail with the mechanical response evoked from normal skeletal muscle by the "close range" injection of small amounts of acetylcholine. The rapidity and general "twitch-like" character of the response to acetylcholine made it appear at first sight that an action potential not grossly different from that

accompanying a motor nerve twitch might be expected. This, however, was found to be incorrect. Leading from belly to tendon, with a degree of amplification greatly in excess of that required to reveal the motor twitch action potential, either failed entirely to show any response when the muscle was excited by acetylcholine, or showed at the best a small, slow, irregular shift of base-line.

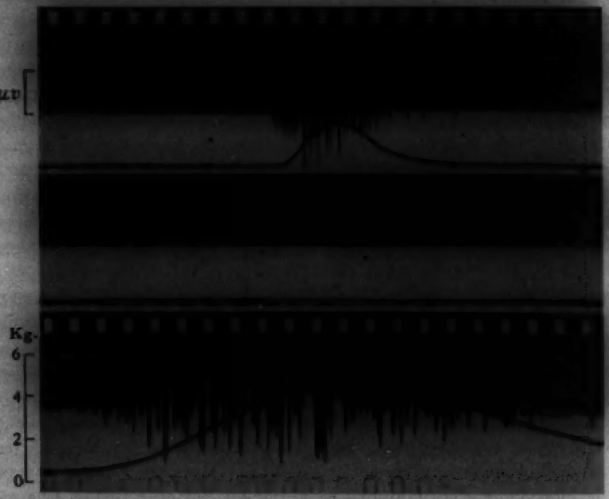

Fig. 2. Cat. Chloralose. Isometric myogram of gastrocnemius. Action potentials with concentric needle electrodes. Effect of close arterial injection of (*a*) 5γ ACh., (*b*) 0·5 c.c. acid saline, (*c*) 25γ ACh. 2 hours later. Higher speed recording surface. In this figure and all subsequent optical records the time record shows intervals of 10 msec.

It was obvious, therefore, that the acetylcholine contraction, quick as it was, either had no accompanying electrical activity, which was unlikely, or that the contractions of the individual fibres were so out of phase as not to appear as a recognizable potential. Accordingly the lead from the muscle fibres was restricted by employing the concentric needle electrode.

Under these conditions of recording, injection of acetylcholine produces an irregular outburst of activity shortly preceding and during the mechanical response; a similar injection of saline at the same *p*H as

Fig. 3. Cat. Chloralose. Action potentials of single muscle fibres produced by arterial injection of ACh. Twin wire electrodes. (*a*) 0·5γ ACh. (*b*) and (*c*) Continuous record of response to 2·5γ ACh.

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Fig. 11. Cat. Decerebrated. Myogram of gastrocnemius. Action potentials with concentric needle electrode. (*a*) Response to nerve volley. (*b*) Response to 20γ ACh. by close arterial injection and to superimposed nerve volley. (The action potentials of the responses to the nerve volleys are obscured by the stimulation artefacts.)

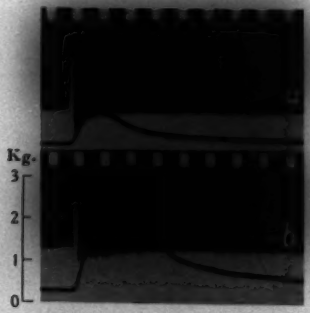

Fig. 12. Cat. Decerebrated. Myogram of tibialis anticus. Action potential, belly-to-tendon lead. 2 hours 20 min. after eserine. Response to single maximal nerve volley, (*a*) before and (*b*) 1 min. after intravenous injection of 1 mg. ACh.

used for the acetylcholine has no such effect (Fig. 2 *a* and *b*). A similar response with a higher speed of recording surface is shown in Fig. 2*c*. These records establish beyond question that the contraction of normal mammalian muscle evoked by acetylcholine is a propagated disturbance in the muscle fibres, set up irregularly throughout the body of the muscle as the acetylcholine reaches the sensitive region of each fibre in succession. There can be no question of a contracture analogous to that occurring in denervated mammalian muscles and in normal muscles of birds and amphibia. That the individual muscle fibre responds more than once during the flooding of the muscle with acetylcholine is clear from the fact that tensions very greatly in excess of the maximal synchronous motor twitch can be produced, by injection of higher concentrations of acetylcholine. The increased localization provided by the concentric needle electrodes does not, however, permit of the detection of the frequency of the response of individual fibres, since the asynchronism of the contraction of the muscle is between fibre and fibre, and concentric electrodes of the usual size do not give a localization more precise than to a single motor unit, consisting of some 100 fibres. Accordingly a number of records have been made using the twin wire electrodes, which, under favourable conditions, allow of the picking up of the responses of individual fibres. Records obtained in this way are shown in Fig. 3 (Plate I). With these leads, shielding of the preparation has to be as complete as possible, and this precluded the simultaneous recording of the mechanical response of the muscle. The most satisfactory records have been obtained with relatively small doses of acetylcholine, and usually after a number of injections had been made; the frequent repetition of saline injections produces some degree of œdema among the muscle fibres and this, presumably, allows the action potential of a single fibre more easily to be picked out among the large number of excited fibres. There can be little doubt that the actual potentials recorded are those of single muscle fibres. The individual spikes of any one series remain at exactly the same amplitude and start and stop suddenly, but during their discharge the frequency falls along a smooth curve of characteristic shape; it is very improbable that the random excitation of fibres or groups of fibres by slowly diffusing acetylcholine would produce this constancy in amplitude and frequency. In Fig. 4 is shown graphically the frequency of response of single fibres taken from Fig. 3*b*. 70 msec. after the first irregular deflexion two discrete spikes appear 5 msec. apart. There is then an irregular discharge followed, 150 msec. from the start of the response to the injection, by regular recurring spikes of constant amplitude. This stops suddenly after

some 90 msec. and is followed by the regular discharge of another, more distant fibre, which continues for 1·2 sec. The discharge of this fibre overlaps the last three discharges of the previous fibre and it may be

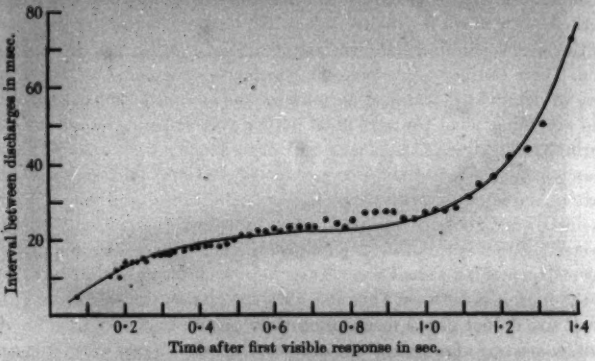

Fig. 4. Curve showing the interval between the responses of single muscle fibres during stimulation by ACh. Taken from Fig. 3 (*b*) and (*c*).

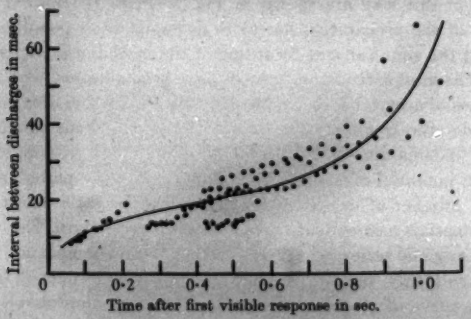

Fig. 5. As Fig. 4. Composite curve from three separate injections of ACh.

noted that the frequencies coincide. The curve shown in Fig. 4 appears to be characteristic of the response to acetylcholine. In Fig. 5 is shown a composite curve obtained from three separate injection responses taken at different times in the same experiment. The general form is much the same as in Fig. 4.

Effect of eserine on muscular response to single maximal motor nerve volleys

After the intravenous injection of doses of eserine of about 0·3 mg. per kg. there is, as Brown *et al.* showed, a gradually increasing potentiation of the muscular response to a single maximal motor nerve volley. The effect is maximal in about 5–10 min. Examination of the action potential simultaneously recorded from the muscle by belly-to-tendon leads shows that during this potentiation the main negative spike is followed by a series of small waves which are visible as discrete deflexions for as long as 60 msec. after the spike. The successive deflexions diminish progressively in size, the base broadens and the interval between consecutive discharges gradually lengthens until nothing more than a slight irregularity of the base-line remains [see Brown *et al.* Figs. 12 and 13]. The progressive diminution in amplitude and the broadening of the base obviously indicate that the discharges of individual fibres are falling more and more out of phase.

A restriction of the lead to a smaller number of muscle fibres by means of concentric needle electrodes allows the repetitive discharge to be followed more precisely. A record taken in such a manner is shown in Fig. 8. It is clear that there is a progressive increase in the interval between consecutive responses, an increasing asynchronism among the individual fibres comprising the unit and, apart from the first few deflexions, a steady decrease in the total E.M.F. developed. The apparent larger amplitude of the deflexions immediately succeeding the first spike is due to their summing with the negative artefact produced by the concentric electrodes and the small coupling condensers.

A number of measurements have been made of the frequency of the discharge. The shortest interval recorded between the first deflexion and the next was 1·7 msec. This was observed at the maximum of the mechanical potentiation in a cat which had received 0·3 mg. of eserine per kg. With smaller doses of eserine, or when the effects of a large dose are wearing off, the interval between the first two responses may lengthen to as much as 4·0 msec. The interval between the succeeding spikes shows a small progressive increase until the response becomes so asynchronous as to prohibit accurate measurement. The interval between two successive responses in relation to the time after the stimulus is shown graphically in Fig. 6. It must be noted that measurement is only possible of the intervals between the crests of the main deflexions and that this can, at the best, give only a value of the mean frequency of a large number of

discharging fibres, the measurement becoming more inaccurate as the temporal dispersion among the fibres increases. Attempts to confine the leads to single muscle fibres, as was done with the acetylcholine injection,

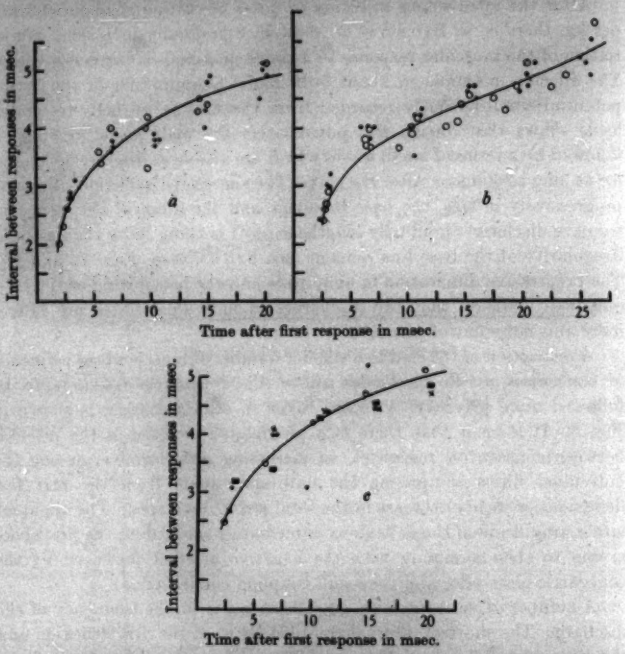

Fig. 6. Curves showing interval between responses in repetitive response of muscle after eserine: (*a*) Dots (·) response to single maximal volley. Circles (o) response to two volleys 1·6 msec. apart. 6 min. after eserine. (*b*) Dots (·) response to single maximal volley. Circles (o) response to two volleys 1·6 msec. apart. 20 min. after eserine. (*c*) Dots (·) response to single volleys. Circles (o) response to two volleys 1·6 msec. apart. Crosses (×) response to two volleys 4 msec. apart. Squares (□) response to two volleys 8 msec. apart. 27 min. after eserine.

proved abortive, probably on account of the fact that, although the discharge becomes asynchronous, there is always sufficient activity throughout the muscle to confuse the record of a single fibre. In most experiments, the measurable, synchronous, repetitive discharge did not

last much more than 30–40 msec., but in one experiment discrete spikes were visible 100 msec. after the stimulus. This provided a graphic record of the later stages of the response not usually visible (Fig. 7). This curve bears a striking resemblance to the curve of the frequency of the fibres after injection of acetylcholine (Figs. 4 and 5), although there is, of course, a big difference in the values of the coordinates. As the experiment proceeds, and the effects of eserine pass off, the interval between the initial

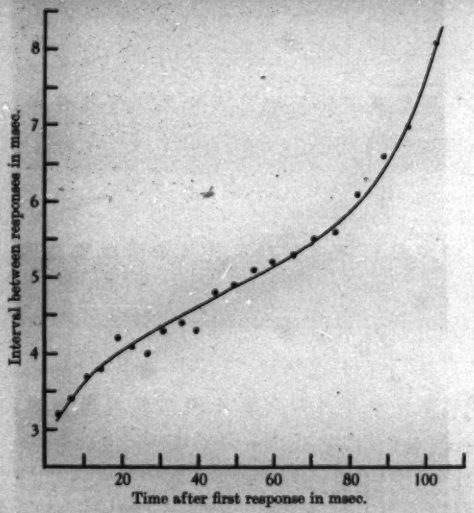

Fig. 7. Curve showing interval between responses in long-lasting repetitive response of muscle to single maximal nerve volley after eserine.

responses lengthens and there is some flattening of the curve. This effect does not appear to be due entirely to the diminution in the concentration of eserine circulating, since a second dose does not increase the frequency but serves apparently only to increase the number of the synchronous responses.

Effect of two maximal volleys

After the usual dose of eserine, and when this has attained its maximum effectiveness, a second volley set up in the nerve during the synchronous repetitive discharge is without effect (Fig. 8). The myo-

graphic tension is the same as that produced by a single volley and no significant alteration is to be observed in the frequency, rate of decline or duration of the repetitive response. In Fig. 6 are shown three curves from one experiment. In the first (*a*) the dots show the frequency curve

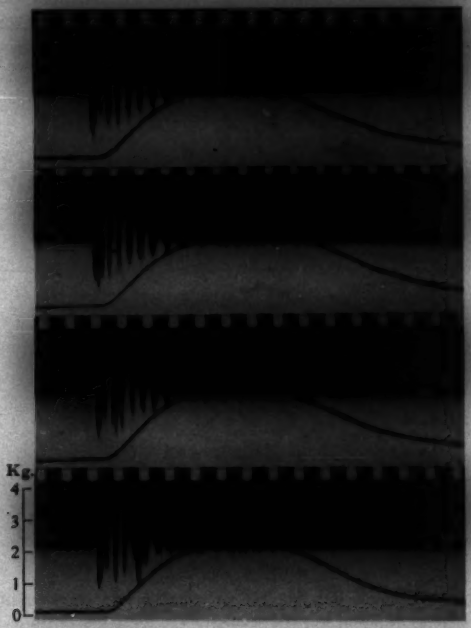

Fig. 8. Cat. 2·4 kg. Decerebrated. Myogram of tibialis anticus. Action potential with concentric needle electrodes. 27 min. after 0·72 mg. eserine intravenous. Responses to maximal nerve volleys. (*a*) Single volley. (*b*) Two volleys 1·6 msec. apart. (*c*) Two volleys at 8 msec. (*d*) Two volleys at 16 msec.

in response to single volleys, and the crosses the response to two volleys set up 1·6 msec. apart. The records from which these points were obtained were taken 6 min. after the administration of eserine. The tensions developed in response to single volleys increased from 2·05 to 2·35 kg., whereas the double volleys produced tensions between 2·4 and 2·58 kg. There is clear evidence from the curve that the interval between the first

and second responses is shortened by the second volley. In conditions such as these the accelerated second response frequently has a narrower base than the second response occurring after a single volley only, indicating the production of a greater synchronization among the fibres. The second curve (*b*) was taken 20 min. after the administration of eserine and there was only a difference of 0·1 kg. between the tensions in response

Fig. 9. Cat. 2·7 kg. Chloralose. Myogram of tibialis anticus. Action potentials led from belly to tendon. One hour after eserine 0·9 mg. intravenous. Responses to maximal nerve volleys. (*a*) Single volley. (*b*) Two volleys 35 msec. apart. (*c*) Two volleys at 50 msec. (*d*) Two volleys at 200 msec.

to the double and the single volleys. This absence of effect of the second volley is reflected in the frequency curve, there being no significant difference between the two sets of points. In the third curve, single volleys and two volleys at 1·6, 4 and 8 msec. apart were used, the record being taken 27 min. after eserine. The double volleys produced no increase in tension over the single.

As the interval between the volleys is increased, usually when it reaches about 30 msec., there appears, in response to the second volley,

a visible action potential which is much reduced in size and is followed by no recrudescence of the repetitive activity. As the volleys are separated further the second action potential increases, a visible prolongation of the myogram is seen and, 200 msec. after the first, the second volley causes definite repetitive discharge after its main action potential (Fig. 9). When the first volley is reduced to such a size that little or no repetitive discharge follows, the depression of a subsequent maximal volley can be followed in greater detail. The depression of the second is apparently maximal when the two are separated only enough to allow

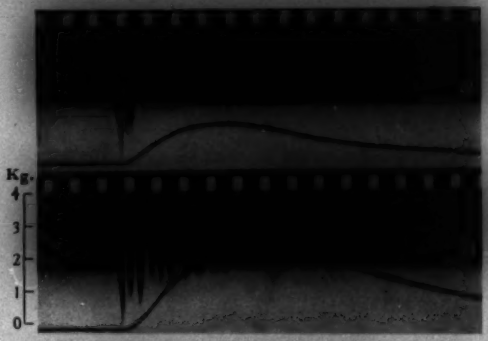

Fig. 10. Cat. 2·6 kg. Decerebrated. Myogram of tibialis anticus. Action potential with concentric needle electrode. 1 hour 40 min. after eserine 0·8 mg. intravenous. (*a*) Response to single maximal nerve volley. (*b*) Response to two volleys 1·2 msec. apart.

the second to appear as a discrete deflexion, viz. about 8 msec. Separation of the two volleys leads to a steady increase of the second, until some 200 msec. after the first it has reassumed its original amplitude. It is of interest that the total tension produced by the small volley and the succeeding depressed response is equal to the tension produced by the maximal volley alone.

When a submaximal dose of eserine (0·05–0·1 mg. per kg.) is given, or the effects of an originally maximal dose are passing off, a maximal volley does not occlude a subsequent volley, but the two summate to produce an effect similar to that of one volley during full eserine action. Fig. 10 gives an example of such an effect. One hour and 40 min. after the administration of eserine (0·3 mg. per kg.), a single volley produced a response little different from that before eserine, but two volleys set

up 1·2 msec. apart gave rise to a full repetitive response with a myographic tension greatly out of proportion to the summed effect of two such volleys before eserine.

Effect of acetylcholine on response to single volley

Brown *et al.* [1936] showed that the injection of a stimulating dose of acetylcholine did not materially affect the response of a muscle to a maximal volley falling 10 sec. after the injection, if the disappearance of acetylcholine from the muscle was not impeded by œdema or the presence of eserine. We have now tested the effect of a stimulant injection of acetylcholine on the response to a volley timed to fall during the acetylcholine contraction. The method employed was to arrange a knock-down key so that, at the moment of depressing the syringe piston, a circuit was opened which released the Lucas pendulum delivering a break shock to the nerve. It was found that the response to a volley set up so close to the acetylcholine contraction as to fall during the decline of tension was not significantly altered (Fig. 11, Plate II). It must be admitted that the measurement of the tension developed is inaccurate, since the twitch superimposed on the acetylcholine contraction starts at a different initial tension; but, as different initial tensions do not affect the response of gastrocnemius very greatly, and a small diminution in tension is to be expected, on the assumption that some fibres are actually refractory at the moment of nerve stimulation, the conclusion appears to be justified that the response to the volley is, at least, not depressed.

In the presence of a large dose of eserine, injection of an excitant dose of acetylcholine into the muscle produces, as noted above, a severe depression of the response to subsequent nerve volleys and a similar effect on subsequent volleys is produced by a nerve volley after eserine. If the actions of eserine are in fact due to its effect in causing persistence of liberated acetylcholine, then it might be expected that a *small* concentration of acetylcholine, insufficient either to excite or depress, circulating through a muscle might produce an effect analogous to that of eserine. This has proved to be the case. For instance, a cat (2·8 kg.) had received an intravenous injection of 2·2 mg. of eserine in all, in two successive doses. Two hours and 20 min. after the second injection, the effects of the eserine had nearly completely passed off. The tension developed in response to a maximal motor nerve volley had declined until it was only 9 p.c. greater than before eserine, and repetitive discharge was no longer visible. The cat then received 1 mg. of acetylcholine intravenously and stimulation of the motor nerve was continued at

10 sec. intervals. After 1 min., the tension in response to a single maximal volley had increased to 2·05 kg. as compared with 0·85 kg. before the acetylcholine. Simultaneously a typical repetitive discharge of the muscle was observed, the action potentials appearing exactly like those after a full dose of eserine (Fig. 12, Plate II). The effect gradually passed off and in some 5 min. the contractions were once more of the simple non-repetitive character. The effect is observable equally well after removal of the suprarenals.

Discussion

In a preceding paper [Brown *et al.* 1936], discussion of the manner in which acetylcholine, given by close arterial injection into a normal muscle, will reach and stimulate the separate muscle fibres led to the view that the quick response of the muscle to such an injection would have the nature of a brief, asynchronous tetanus. Electrical records have now demonstrated the correctness of this deduction. Records from single muscle fibres have shown that the response starts at a frequency of some 200 per sec. and then gradually declines. There is no reason to doubt that these responses are anything but "All-or-Nothing", propagated, disturbances initiated at the motor end plate. Brown *et al.* [1936] showed that a contraction of this type is not followed by any depression of the response of the muscle to a single volley following some 10 sec. afterwards, if the rapid disappearance of acetylcholine is not prevented by œdema or eserine. This observation has been confirmed and extended to show that the effect of a maximal volley during the acetylcholine contraction is likewise unaffected. In the paper mentioned above, and in a subsequent one by Bacq & Brown [1937], evidence was given that when eserine is present, acetylcholine, whether liberated by impulses at the nerve endings or injected into the blood vessels, has a depressant after-effect on the response to further nerve impulses. Brown *et al.* suggested that this depressant action was exerted, not on the motor end plates, but on the general body of the muscle fibres, attributing it to the lingering of acetylcholine in the interstitial spaces. Evidence which I have here put forward makes it unlikely that such an action, if present at all, will account for all the phenomena of depression. Records such as that of Fig. 3 give clear evidence of a stimulation of the end plate of a muscle fibre by acetylcholine lasting certainly more than 1 sec. This must be pictured as due to acetylcholine persisting in the paramuscular spaces, from which it maintains, by diffusion, a stimulant concentration at the muscle end plate, where it excites and is immediately destroyed by a local concentration of cholinesterase, and where, failing such renewal,

it would immediately fall below stimulating strength. Yet under these conditions, in which a persistence of acetylcholine in contact with the general body of the muscle fibre must be postulated, no depression is produced of the response to renewed stimulation by a nerve volley. It is only when eserine is present in sufficient strength to weaken this action of the cholinesterase concentrated at the nerve ending that the after depression comes into the picture. The meaning of this depression, and the nature of the excitable structures which it involves, are at present being further investigated.

The hypothesis that the repetitive response of the muscle fibre to a single nerve impulse after eserine is due to the persistence of acetylcholine has received further support from these experiments. The introduction into the circulation of amounts of acetylcholine, in themselves insufficient to excite the muscle directly, has been shown to evoke the typical, repetitive response of the muscle produced by a full dose of eserine. Furthermore, it has been shown that when insufficient eserine is present to evoke a repetitive muscular response, two volleys set up close together in the nerve can produce peripherally a summation, which is evidenced by the appearance of long-lasting repetitive contractions. The fact that this phenomenon can be observed when the volleys are separated by as much as 15 msec. makes unfeasible any explanation of the eserine effect based on an augmented irritability of the muscle fibre.

The data obtained of the time relation of the multiple response of the muscle to a single nerve volley after eserine are in complete consonance with the conception of the liberation at the nerve ending of a charge of acetylcholine which, normally destroyed during the refractory period, can, after eserine, persist and re-excite the fibres as they recover from their refractory periods. The shortest time at which the second response in a repetitive series has been found to occur is 1·7 msec.; under the conditions of these experiments, the shortest time for muscular summation, in the absence of eserine, has been found to be 0·8–1·0 msec. with the stimulating electrodes some 5 cm. from the muscle. Allowing for the delay of the second impulse, travelling in a partially recovered nerve, the figure of 1·7 msec. must be very close to the absolute refractory period of the neuro-muscular system. The visible synchronous repetitive contractions of the muscle last for some 30 msec. after the first response, and considerable irregular activity can often be seen as long as 0·5 sec. afterwards. It is reasonable to assume, therefore, that the amount of acetylcholine liberated at the nerve ending is greatly in excess of the minimal amount required to set up an "All-or-Nothing" response in the muscle

fibre and that, in normal conditions, either the end plate receives a greatly supraliminal stimulus or, that much of this acetylcholine is actually inactivated by esterase as it is liberated.

That the decline in frequency and ultimate cessation of the discharge is to be attributed entirely to the fall of the liberated acetylcholine below the stimulating concentration is open to question. The whole phenomenon is undoubtedly complicated by the depressant effects of the persisting transmitter. A second volley only produces a visible action potential when separated by some 30 msec. from its predecessor and, as Bacq & Brown have shown, successive stimuli so far apart as 5 sec. may eventually fail to give a full response. The failure of a volley following another at such short time intervals as 1–15 msec. to show any separate action potential or to affect materially the mechanical response is understandable, as such a volley probably falls upon refractory muscle fibres, but the failure of such a volley to alter, in any way, the duration and frequency of the repetitive discharge cannot at present be explained. The phenomenon is part of the complex picture of the depressant effects of acetylcholine and full discussion must be deferred until more facts are available.

Summary

1. Records of action potentials from normal mammalian muscle have shown that the quick contraction evoked by the close arterial injection of acetylcholine is a brief asynchronous tetanus.

2. The components of this contraction are propagated "All-or-Nothing" responses, starting at a frequency of some 200 per sec. and then gradually falling in frequency along a characteristic curve.

3. The effect of eserine in producing a repetitive response of mammalian muscle to a single maximal nerve volley has been investigated in greater detail. The initial frequency of the response has been found to be such that successive contractions fall close to the absolute refractory period of the muscle fibre.

4. A second nerve volley, following closely after one which has produced the full repetitive response of the muscle, is without effect if the interval between the volleys is less than 20 msec. Further separation of the volleys produces a gradual recovery of the effect of the second.

5. In the presence of a dose of eserine, insufficient in itself to cause a repetitive response of the muscle to a single nerve volley, the effects of two suitably timed volleys undergo summation, and evoke a repetitive response.

6. The persistence in a weakly eserinized muscle of acetylcholine, in a concentration insufficient in itself to cause any response, causes the muscle to give a repetitive response to a single nerve volley.

I wish to thank Sir Henry Dale for his stimulating interest in this investigation.

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THE ELECTRICAL RESPONSES OF LIGHT-ADAPTED FROGS' EYES TO MONOCHROMATIC STIMULI

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After adaptation to bright sunlight the frog's retina is still capable of responding to strong stimuli with a definite electroretinogram. This, no doubt, must be set up by an initial reaction in the cones which we shall define as active elements lacking visual purple [Granit, 1936]. Two facts support this conclusion: (i) the practically pure rod eye of the white rat gives no electrical response in the light-adapted state [Charpentier, 1936], and (ii) the "mixed" eye of the frog with an approximately equal number of rods and cones shows an electrically measurable Purkinje shift [Himstedt & Nagel, 1901, confirmed by others]. After light adaptation the maximum of the initial *b*-wave of the frog's electroretinogram shifts towards the red end of the spectrum. But the extent of this shift in sensitivity and the precise nature of the curves relating size of *b*-wave to wave-length for an equal energy spectrum has not yet been determined.

Continuing the work of Granit & Munsterhjelm [1937] in which the *b*-waves were measured in the dark-adapted frog's eye, we have now with the same apparatus obtained a similar body of data for the light-adapted preparation. The measurements have also been planned to elucidate the interesting question concerning a possible "specific effect of wave-length" on the electrical response.

Now there can be no doubt about the fact that the frog's eye shows a specific effect of wave-length. This was described by Gotch [1904], by Brossa & Kohlrausch [1913], and more recently by Smit [1934] and Granit & Munsterhjelm [1937]. But since rods and cones separately give rise to *b*-waves, the specific effect of wave-length can be due (i) either to different types of cones being selectively sensitive to different spectral regions, or (ii) to rods and cones giving somewhat different types of responses overlapping in certain parts of the spectrum. Only in the former

case does the specific effect of wave-length assume any significance for colour theory. In the frog's eye the rods alone do not react specifically to wave-length [Granit & Munsterhjelm, 1937; for work on other eyes see Kohlrausch, 1931; Graham & Riggs, 1935].

It is necessary at the outset to realize how a specific effect of wave-length might be demonstrated. Kohlrausch and Brossa have devoted several papers to this question [Brossa & Kohlrausch, 1913; Kohlrausch & Brossa, 1914; Kohlrausch, 1918] and with the pigeon representing one type of eye find that, no matter how intensity of the stimulus is varied, it is impossible to obtain a match between responses elicited by long and short wave-lengths. These experiments have recently been repeated by Graham *et al.* [1935] who have come to quite a different conclusion that, merely by adjusting the intensity of the stimulus, responses from the two ends of the visible spectrum can be made identical.

It is also possible to approach the problem in a slightly different and more satisfactory manner, applied by Brossa & Kohlrausch [1913] in their first contribution to this problem. This is to use equipotential responses from the red and blue side of the spectrum and find out whether in every respect they match. If they do, the method is not applicable to a study of the hypothetical selective sensitivity to different wave-lengths, as identical responses may well be given by, say, photochemical substances with different absorption curves. But if the electroretinograms obtained do not match, except in height of potential, then the equipotential responses certainly are given by different elements or combinations of elements. The chief experimental difficulty is to avoid mixed rod and cone responses due to dark adaptation. Thus in the frog's eye equipotential responses do not always match, but extensive measurements led Granit & Munsterhjelm [1937] to the conclusion that, at least in the dark-adapted eye, such cases are due to rods and cones overlapping. In our opinion the other "true" type of specific effect of wave-length has not yet been convincingly demonstrated for any eye (cf. Graham *et al.* [1935] criticism of the best supported claim in the case of the pigeon).

Technique

A detailed description of the technique has been given by Granit & Munsterhjelm [1937]. The same string galvanometer, amplifier and Tutton Monochromator were used, the experiments with light-adapted frogs being carried out on sunny days in April, May and June with twenty-seven frogs (*R. esculenta*) obtained from Hungary in March. Frogs from the same group were used in work with dark-adapted eyes

when the sky was cloudy. The animals to be light adapted were first dark adapted for not less than 12 hours. Apart from minor differences of procedure, to be mentioned below, the high intensity curve of Granit & Munsterhjelm [1937]—their Fig. 4—and our standard curve for light-adapted eyes (Fig. 4) have been obtained in the same manner. In order to obtain the latter curve it was only necessary to raise the intensity of the spectral light by 1·3 logarithmic units (removal of the "auxiliary density", mentioned in the work of Granit & Munsterhjelm to which all our comparisons with scotopic eyes refer, unless otherwise stated). Wave-length 0·560 μ was used as a control of the general level of sensitivity of the preparation. The other readings are given in percentage of the value at 0·560 μ.

Light adaptation was secured by leaving the glass jar containing the frog for an hour in a sunny window. Then the eyes were removed, padded in cotton soaked with Ringer, and left for a further 10–15 min. in the window. The excised eye was moistened every now and then. The pupil of the light-adapted, intact frog is relatively wide, though, of course, smaller than in the scotopic state. It contracts during the operation, and again dilates when the eye is left undisturbed in the sunshine. Further dilatation takes place in the dark box. In some experiments atropine was tried, but it was soon found to be of little use, as was to be expected.

A great number of experiments were carried out with opened bulbs, and in these cases the bulbs, filled with Ringer, were left for only 5–10 min. in the window after the standard exposure of 1 hour of the intact frog. Several eyes were repeatedly light adapted so that in most cases a fairly large number of observations was available for an eye in good condition.

The readings were taken at a rapid pace, usually with one, sometimes with half a minute between exposures lasting 1 or 3 sec. With good preparations ten exposures of a given wave-length at this rate could be identical within the errors of measurement. The onset of dark adaptation was recognized by several signs, to be mentioned separately below. Sometimes dark adaptation was allowed to proceed, sometimes the eye was readapted to light and the experiment repeated. According to Kühne [1879] visual purple may be detected after some 20 min. of dark adaptation. Actually this is roughly the time within which the responses are free from an admixture of rod reactions (rods being defined as active elements possessing visual purple). But in our case this presupposes that the eye is repeatedly stimulated with a bright light. The well-known optograms of Kühne demonstrate that illumination of a given area leads to local bleaching of visual purple.

Often the retinae were removed after the experiment and inspected. Generally the colour was a pale yellow or red, but then most experiments were carried well beyond the point when signs of dark adaptation occurred. When immediately after light adaptation the second eye was removed and its retina inspected it was always found to be colourless. No oxygen was used in these experiments [cf. Granit & Munsterhjelm, 1937] as it was suspected of furthering regeneration of visual purple.

Results

General observations

As long as the bulb is intact, the values for size of *b*-wave against wave-length fall on a very definite and regular curve. Diphasic *b*-waves have been noted with the smaller responses towards the end of the spectrum but they have been exceptions, and not the rule, as in dark-adapted eyes. With opened bulbs we have sometimes had indications of a switchboard effect of the type encountered with the scotopic preparation, but though responses to a constant stimulus have changed in size, they have not differed in type as definitely as in the scotopic eye. As diphasic responses have also been seen by Meservey & Chaffee [1927] in the cone eyes of the horned toad and the chameleon, we have every reason to assume the *b*-wave to be multiple in cone eyes. The difference in this respect between the lability of the scotopic eye and the stability of the photopic preparation merely indicates that in the latter case we worked near the threshold, whereas with the light-adapted eye we used relatively strong stimuli. As will be shown below, weak stimuli could not very well have been used.

Another difference between light- and dark-adapted eyes probably has some physiological significance. This is the fact that with the scotopic retina the distribution of sensitivity to the spectrum varied a great deal from eye to eye, whereas the light-adapted retina is a fairly stable preparation until dark adaptation sets in and alters the picture.

Recognition of dark adaptation

Dark adaptation is heralded by several signs to which a great deal of attention must be devoted. Unless it can be regarded as demonstrated beyond doubt that a given type of eye is lacking rods containing visual purple, the question as to whether or not some regeneration of visual purple has taken place must be settled with the aid of definite criteria before a given result can be attributed to the cones alone. The stimuli are

supermaximal for the visual purple system which probably is activated as soon as traces of visual purple appear.

In the dark-adapted eye the *b*-wave is of maximal size between 0·500 and 0·510 μ and the response at 0·560 μ is at about 68 p.c. of the maximum. We shall see below that after light adaptation the maximum is at about 0·560 μ [cf. Smit, 1934], and the response at 0·510 μ is about 72 p.c. of the maximum. In accordance with this fact, the general change indicating the onset of dark adaptation is a shift in sensitivity making the short wave-lengths relatively more effective.

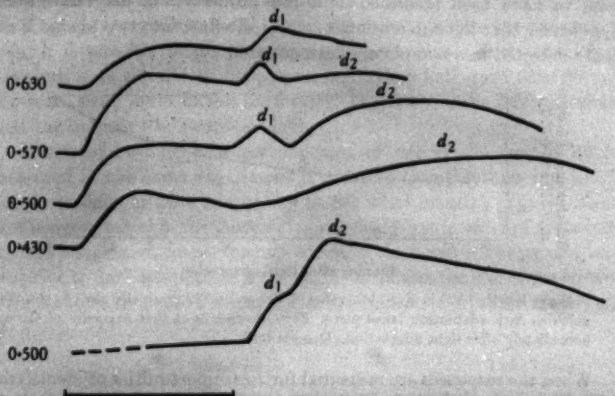

Fig. 1. Series of four responses to 1 sec. flashes at the standard intensity, illustrating diphasic off-effects. The lowest curve shows end of 3 sec. stimulus. *Note*: Only first phase of off-effect at 0·630 μ (uppermost curve), second phase already present but small at 0·570, a great deal larger at 0·500, only second phase visible at 0·430. The lowest response at 0·500 also shows diphasic off-effect, but owing to the long exposure the off-effect larger than in the other curves. Time below 1 sec.

During the course of the experiment the following five criteria are the most useful ones: (i) the appearance of a secondary rise (*c*-wave); (ii) the responses around 0·500–0·510 μ become relatively too large; (iii) the off-effect from having been rapid and monophasic often becomes diphasic, an initial, small and rapid phase, and a second slow rise (see Fig. 1), part of which may be a *c*-wave; (iv) when the responses at 0·620 and 0·470 μ are compared the secondary slow rise of the *c*-wave and the similarly slow second phase of the off-effect are found to be absent at 0·630 μ and large at 0·470 μ at a time when the difference in the *b*-waves has not yet clearly

developed; (v) the changes mentioned above are progressive. In the best eyes all these changes are to be seen simultaneously.

The secondary rise and the second phase of the off-effect are identified as rod phenomena by following the standard curves for size of *b*-wave against wave-length which characterize the rods [Granit & Munsterhjelm, 1937] and also with the aid of the very convenient criterion (iv). Long ago v. Brücke & Garten [1907] showed that in the frog's eye the *c*-wave disappears after light adaptation. Diphasic off-effects have also been seen before, e.g. by Smit [1934], but the second slow phase does not seem to have been identified as a rod property. It may have some significance that the differentiation of the off-effect into two phases is not easily detected in a completely dark-adapted eye.

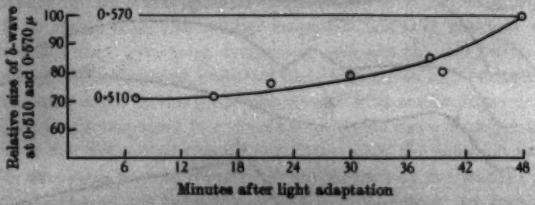

Fig. 2. Wave-length 0·570 is given the value of 100 and 0·510 gradually rises to the same value as dark adaptation takes place. Zero abeciasa is at first exposure of the eye immediately after light adaptation. Opened bulb.

When the responses are measured further opportunities of identifying rod activity arise. Thus the rate of rise of the *b*-wave becomes relatively faster towards the short wave-lengths, as is found in the completely dark-adapted eye at high intensities of stimulation. The maximum of the curve for the *b*-waves (see Fig. 4) expands, particularly towards the blue. The values in the red then become relatively too small. All these effects can be predicted from the known properties of the rod system of this eye on the assumption that rod responses are superimposed upon cone responses [see Granit & Munsterhjelm, 1937] and need not be further discussed.

The time course of the changes indicating dark adaptation have raised some interesting points. The usual expansion of the maximum is shown in Fig. 2. This is from an experiment in which the eye had been readapted to light, having already served once for a series of measurements at different wave-lengths. The wave-lengths 0·570 and 0·510 μ were taken alternately, and after some ten or twenty readings the time was noted and the string calibrated. The observations preceding each calibration were

averaged and have been marked in the diagram as referring to the moment of calibration. The *b*-wave at 0·570 μ has been given a constant value of 100 and the diagram shows how the *b*-wave at 0·510 μ rises to become identical with the reading at 0·570 μ after 48 min. During the first 20 min. their mutual relationship remains practically unchanged. These time intervals are typical of most experiments, but there have been exceptions. With one eye we succeeded in taking ninety-five observations, all of which fell on much the same curve for size of *b*-wave against wave-length. The averages differed by a mean variation of only 2·8 p.c. from the final averages for the light-adapted eye shown in Fig. 4.

Whereas observations of this type show that, despite the presence of rods, it is possible to isolate another element with a fairly stationary distribution of sensitivity to spectral light, they hold good only for size of *b*-wave as an index of this sensitivity. They require the further proviso that the intensity be relatively high.

If size of *b*-wave, off-effect and rate of rise of the *b*-wave all be measured in the same experiment it is found that the three indexes do not show signs of dark adaptation at the same moment. The off-effects first increase towards the short wave-lengths. Next in order of sensitivity to dark adaptation comes the rate of rise of the *b*-wave. The process least sensitive to dark adaptation is the one determining the size of the *b*-wave. This is shown in Table I where the initial values are given to the left and

TABLE I

	Initial values						Later values					
Wave-length μ ...	0·450	0·490	0·500	0·530	0·580	0·650	0·440	0·470	0·500	0·540	0·600	0·640
Order of observation	5	8	2	6	9	10	16	24	13	25	15	19
Rate of rise of *b*-wave	28	47	70	92	74	33	28	53	63	114	—	—
Size of *b*-wave	20	38	65	87	92	32	20	41	63	89	70	33
Size of off-effect	39	82	76	89	85	32	73	105	112	116	48	33

All values are given in percentage of reading at 0·560 μ.

later values to the right with the figure for the order of the observation in the upper horizontal row. The later *b*-waves do not differ very much from the earlier ones, but "rate of rise" and "size of off-effect" have increased a great deal in the short wave-lengths, the latter values already being distributed as in a dark-adapted eye. All values are given in percentage of the reading at 0·560 μ.

The danger of using too low an intensity is illustrated by Fig. 3, representing the result of an experiment in which alternating observations at a high and at a low intensity were obtained by removing and replacing a filter. The low intensity (circles) was the one used by Granit &

Munsterhjelm [1937] for their high intensity scotopic curve. The high intensity is the standard stimulus used in the experiments presented in Fig. 4. It is obvious that the readings at the low intensity (circles) show an effect of dark adaptation which is much less marked at the higher intensity (dots). Incidentally the results demonstrate that the maximal intensity, used by Granit & Munsterhjelm [1937], actually was above the cone threshold, as concluded by them from other evidence.

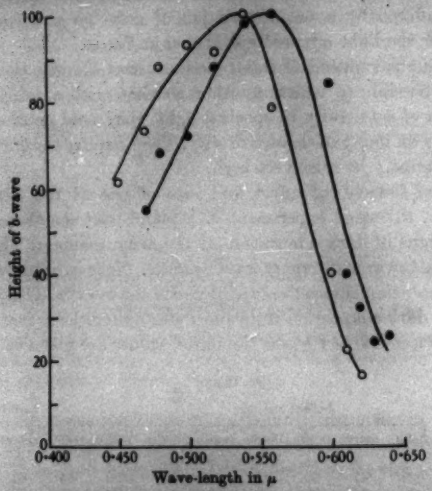

Fig. 3. Size of *b*-wave against wave-length. Circles, low intensity; dots, standard (high) intensity (see text). *Note*: Low intensity curve shifted towards the short wave-lengths. Dark adaptation just beginning.

Relative to an index of sensitivity or "visibility" such as size of *b*-wave dark adaptation may be latent or manifest. Apparently dark adaptation takes place very rapidly, but for some reason or other high intensity *b*-waves do not register this change until a certain limiting value has been reached. Thus size of *b*-wave is a more reliable index of pure cone vision than its rate of rise or the size of the off-effect. Why the off-effect, when present, should be so sensitive to dark adaptation is still a matter of conjecture.

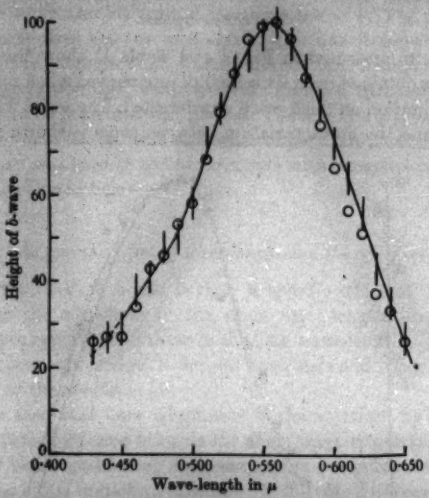

Fig. 4. Average results of 295 observations (six series) showing height of δ-wave, plotted as lines (mean variation doubled), and its rate of rise (circles) against wave-length. Only the former curve drawn. Average potential of δ-wave at 0·560 μ is 0·230 mV.

TABLE II

Wave-length μ	Size of δ-wave in percentage of 0·560	Mean variation
0·420	18	1·5
0·430	24	2·8
0·440	27	4·5
0·450	29	4·3
0·460	38	3·2
0·470	41	3·8
0·480	48	3·8
0·490	50	7·0
0·500	58	5·5
0·510	72	5·2
0·520	80	4·0
0·530	89	1·9
0·540	92	2·4
0·550	97	3·0
0·560	100	—
0·570	95	3·8
0·580	89	3·2
0·590	81	2·8
0·600	72	2·3
0·610	64	3·5
0·620	56	2·5
0·630	43	5·8
0·640	35	4·5
0·650	27	4·3

The cone response

The results presented in Fig. 4 and Table II show the averages of 295 readings obtained from six series, all interrupted when some influence of dark adaptation on the *b*-wave was detected. The series were averaged separately and the mean variation between them is shown in the table.

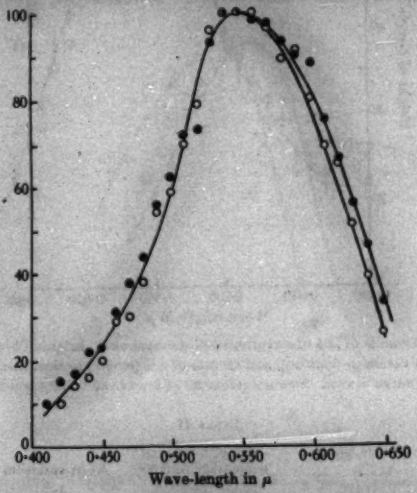

Fig. 5. Dots, size; circles, rate of rise of *b*-wave against wave-length at maximal intensity. Single experiment.

The latter figures indicate maximal lability in the region around the maximum of the rod curve between 0·490 and 0·530 μ. The circles in Fig. 4 refer to rate of rise of the *b*-wave. The curve for size of *b*-wave, drawn through the lines, is symmetrical between 0·490 and 0·650 μ around a maximum at 0·560 μ. From 0·480 μ farther towards the blue and violet the values are relatively too high to fit a symmetrical curve.

The curve for rate of rise of the *b*-wave has not been drawn, but obviously it is identical with the curve for size of *b*-wave except towards the red end beyond about 0·590 μ. Equipotential responses in the red and in the blue therefore differ with respect to dp/dt, the red responses being relatively slower.

Size of *b*-wave and its rate of rise are plotted in Fig. 5 for a still higher intensity than the one used above. This was obtained by trebling the width of the collimator slit. The results are from a single experiment in which the eye was light adapted twice, giving a total of seventy-two observations to be measured and plotted. The maximum is expanded and the values towards the red side somewhat larger than those of Fig. 4. The same intensity was used in the experiments with excessive light adaptation to be mentioned below.

The specific effect of wave-length and the off-effect

The curves of Figs. 4 and 5 show a specific effect of wave-length, inasmuch as the responses in the short wave-lengths reach a given potential more rapidly than those in the long wave-lengths. Is this effect a pure cone property or does it depend upon rods and cones overlapping in this part of the spectrum?

We have seen that dark adaptation is characterized by the *b*-waves becoming relatively faster towards the short wave-lengths and that this change may be noticed before dark adaptation has influenced the size of the *b*-wave as a function of wave-length. If the cone *b*-waves actually are relatively faster towards the short wave-lengths, then this effect should be noticeable in all experiments, and also in the earliest values taken in each series. As a matter of fact this is not the case. There have been individual experiments in which rate of rise and size of *b*-wave are perfectly symmetrical with respect to one another, and the slight specific effect of wave-length in the final averages undoubtedly is due to dark adaptation, latent with respect to size of *b*-wave, but manifest in its rate of rise. The specific effect of wave-length thus belongs to the second of the two types mentioned in the introduction. The section on recognition of dark adaptation shows other instances of the same type of effect, in fact, most of the criteria used for diagnosing dark adaptation depend upon the appearance of a specific effect of wave-length.

If two potentials be otherwise identical but different with respect to the size of the off-effect, this also would represent a specific effect of wave-length and we would have to raise the same questions as above with respect to dp/dt. As a matter of fact all off-effects are relatively too large between about 0·460 and 0·520 μ. Let us as an illustration choose an experiment in which the size of the *b*-wave and its rate of rise were symmetrical with respect to one another and latent dark adaptation thus at a minimum. Even in this case, as shown by Fig. 6, the off-effects are

relatively larger in the green and blue part of the spectrum, and this phenomenon is also present in the earliest observations of this series.

Are we then to assume that we have not succeeded in light adapting so as to remove the influence of dark adaptation on the off-effect, or have

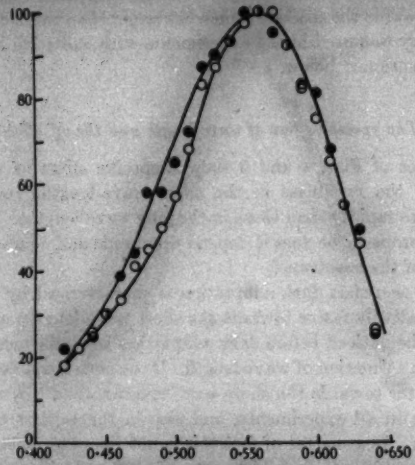

Fig. 6. Size of *b*-wave (circles) and off-effect (dots) against wave-length. Single experiment at standard intensity.

we discovered a true specific effect of wave-length in an isolated cone system? The present results favour the latter explanation, but a final decision in this issue is better postponed till we have more information.

Excessive light adaptation

We have found the initial phases of equipotential cone responses to be identical in every respect independently of wave-length. Rate of rise is the only function carefully measured, but inspection of the curves, the method hitherto considered satisfactory, has not brought to light any other differences deserving mention. Let us assume this to mean that a red and a green of the same potential are elicited by the same group of elements, or, in other words, that the retina—inasmuch as its properties are expressed by its action potential—has no means of sending afferent impulses which are different for "red" and "green". Then, no matter

how the eyes were treated, the red and green responses had a chance of remaining identical, provided that they could still be measured with a reasonable degree of accuracy. From this point of view it would be interesting to try various methods of interfering with the responses. With the experiments on excessive light adaptation we are making a beginning along this line of approach.

The excised, opened bulbs were left in the sunshine for some 5–10 min. Some retinae did not stand this exceedingly strong stimulus, but with twelve preparations the experiments gave definite and measurable results. The eyes recovered later to the extent of dark adapting. As it was impossible to test the immediate effect of excessive light adaptation on the whole spectrum several readings were taken at some wave-lengths and occasional readings at others. Readings were always taken at 0·560, 0·600 and 0·510 μ.

The averaged results for wave-lengths at which the greatest number of observations were made are shown in Table III. Readings from among the first ten following light adaptation are shown in the upper row, whilst later readings obtained before there were any signs of dark adaptation are shown in the lower row. The *b*-waves between 0·560 and 0·610 μ are relatively larger immediately after light adaptation, those around 0·500–0·530 μ relatively smaller. At 0·450 μ the difference between the two sets of values is less definite.

TABLE III

Wave-length μ	0·450	0·510	0·530	0·560	0·580	0·600	0·610
Size of initial *b*-waves ...	30	66	81	100	95	88	70
Size of later *b*-waves ...	33	74	86	100	87	73	65

All values in percentage of reading at 0·560 μ.

The increase in the red and the diminution in the green relative to 0·560 μ have not been equally definite in all experiments. However, no initial value for 0·510 μ has been above 74 p.c., but several readings at 0·600 μ have been between 100 and 90 p.c. Later in the experiment 0·510 and 0·600 μ give approximately equipotential responses, as in the standard curve of Fig. 3, but obviously they are selectively sensitive to excessive light adaptation.

The results throw doubt on the assumption that equipotential red and green responses are elicited by the same elements or combination of elements, and suggest that at least two elements go to make up the symmetrical part of the cone curve.

Blue and violet

The region between 0·480 and 0·400 μ occupies a special place in this as well as in the work with the dark-adapted eye [Granit & Munsterhjelm, 1937]. Quite often the responses were relatively too large in this region in the dark-adapted eye, and in Fig. 4 the same part of the spectrum appears as a kind of an "appendix" to what otherwise would have been

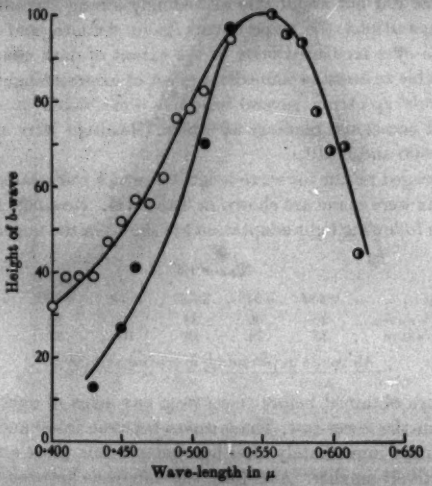

Fig. 7. Height of b-wave in two experiments averaged and plotted against wave-length. Dots, initial values; circles, later values, before dark adaptation has set in. Opened bulbs and maximal intensity.

a perfectly symmetrical curve, reaching a zero value around 0·440 μ. If this had been the case we would have had a curve corresponding roughly to the human curve of the photopic eye similarly plotted. As it is, the responses are relatively too big in this region.

In two experiments with excised opened bulbs the same part of the spectrum reacted in a manner suggesting a common explanation of the irregularities in the two states of adaptation. Some dark adaptation was allowed to take place and the curves, instead of rising considerably around 0·500 μ, as they generally do, rose chiefly towards the blue and violet end as seen in Fig. 7. The narrow curve shows the initial values, the curve

expanding towards the short wave-lengths the later results. At 0·400 μ the response is about 33 p.c. of the maximum. In both these cases the retinae were inspected after the experiment. One retina was yellow, the other one showed a pale reddish colour. It should be noted that the energy of the spectrum between 0·450 and 0·400 μ is uncorrected. Between these wave-lengths the stimuli are relatively too weak and yet the responses are relatively too big.

Some recent observations from work in progress are of interest in this connexion. We have been comparing dark-adapted frogs stored cold (at about 5°) and warm (at about 22°) using weak stimuli. Most of the cold frogs so far have given very large responses in the blue and violet, whereas the warm ones have behaved irregularly, some of them giving small responses in the blue and violet.

Discussion

Our main task in this paper and in the corresponding work with dark-adapted eyes [Granit & Munsterhjelm, 1937] has been, as it were, to survey the ground in order to learn something about the limitations and possibilities of this method as an aid to the study of colour vision. From this point of view we want to draw attention to complications such as the multiple nature of the *b*-wave, the "switchboard effect" [Granit & Munsterhjelm, 1937] and the difficulty of isolating rod and cone responses.

The rod and cone curves are compared in Fig. 8. In the section on recognition of dark adaptation other differences between the electrical properties of rod and cone responses are to be found. The lability of the rod curve is expressed by its double contour and the presence of asymmetrical curves combining inner right and outer left leg and vice versa.

Can we now assume that we have completely succeeded in isolating rod and cone responses? We believe the relatively stable, symmetrical part of the cone curve to represent the maximum degree of isolation obtainable with this eye. As to the rod curve of Granit & Munsterhjelm it is possible that some low threshold cones participate in the reactions towards the red end, owing to the fact that work very near the absolute threshold is made impossible by the switchboard effect. This again we have had reason to note when experimenting with cold and warm frogs. But there remains to be explained the fact that some eyes give very large responses in the blue and violet, i.e. a region in which the absorption curve for visual purple varies between 40 p.c. at 0·450 μ and values near zero at 0·400 (see Fig. 8 of Granit & Munsterhjelm [1937], giving the

absorption curve for visual purple as determined by Dr R. J. Lythgoe). There is also the "appendix" to the otherwise symmetrical cone curve in the same region. A curve published by Chaffee & Hampson [1924] shows exceedingly large responses at 0·400 μ, and Smit [1934] also has noted very large responses in this region in some experiments with dark-adapted eyes. Finally our results indicate a common cause in light

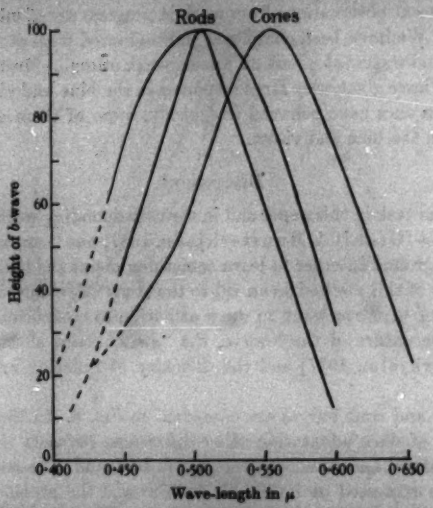

Fig. 8. Rod and cone curves for size of b-wave against wave-length. Rod curve with double contour and maximum at 0·507 μ from Granit & Munsterhjelm [1937]. Cone curve with maximum at 0·556 μ from Fig. 4. Broken lines in region where energy of spectrum uncorrected and stimuli thus relatively too weak.

and dark-adapted eyes, to the irregularities in this region which seems to occupy a special place in our results just by virtue of this property of the responses to blue and violet.

Recent work on the photochemistry of visual purple [referred to by Granit & Munsterhjelm, 1937; see also Hosoya & Saito, 1935; Chase, 1936] suggests that some intermediate product in the chain of bleaching of visual purple [Wald, 1935], as e.g. visual yellow or visual "white", has photochemical properties which would meet the case.

The rest of the cone spectrum is more closely knitted together in a homogeneous symmetrical curve, but the results with excessive light adaptation suggest that this curve is composite and at least contains two elements, a "red" and a "green" which, in order to give the cone curve, would have to overlap within the cone spectrum to a considerable degree.

Most interesting seems to us the fact that even immediately after light adaptation the curves for size of *b*-wave and size of off-effect did not coincide, the latter giving relatively larger responses in the green. "On" and "off" therefore have not been elicited by photochemically identical combinations of elements. It is difficult to believe that this result was due to latent dark adaptation, but the question is important enough to merit a reinvestigation with differently designed experiments. Hartline [1935] has shown that "on" and "off" are partly transmitted by different fibres so that there is nothing inherently improbable in our result, which is also supported by the experiments with excessive light adaptation throwing doubt on the apparent homogeneity of the cone spectrum.

Dr Hartline also has kindly informed one of us that he finds the different fibres of the optic nerve differ greatly in their reactions to monochromatic light. As the various types of *b*-waves are associated with a discharge of impulses through the optic nerve [Granit & Therman, 1935] our results together with those of Granit & Munsterhjelm [1937] demand a minimum of three types of fibres: fibres connected with (i) rods containing visual purple, (ii) rods (or cones) containing a substance absorbing in the blue and violet part of the spectrum ("visual yellow"), (iii) cones. In addition our results suggest that there are at least two types of cones, "green" and "red" cones over which "on" and "off" fibres may be asymmetrically distributed with respect to one another.

Summary

The electroretinograms of light-adapted frogs' eyes in response to monochromatic stimulation with an equal energy spectrum (0·450–0·650 μ) have been obtained with a string galvanometer and a directly coupled amplifier.

Curves showing size of the *b*-wave, its rate of rise and size of the off-effect as a function of wave-length are given in the paper, special precautions having been taken to avoid dark adaptation.

Dark adaptation may be latent or manifest with respect to size of *b*-wave. Its rate of rise and the off-effect are relatively more sensitive

to dark adaptation than the size of the *b*-wave. Dark adaptation often gives rise to a slow second phase of the off-effect, and generally to a *c*-wave.

The initial phases of the electroretinogram show a specific effect of wave-length only after dark adaptation. The isolated cone response does not show a specific effect of wave-length.

The off-effects in the green are relatively larger than the *b*-waves of the same region, even when care has been taken to avoid dark adaptation.

Excessive light adaptation depresses "red" less than "green".

This work supplements that of Granit & Munsterhjelm [1937] with the dark-adapted eye. Together the two sets of results show that there must be at least three substances, retinal elements and types of fibres in the eye: (i) rods containing visual purple, (ii) rods (or cones) containing a substance absorbing light in the blue and violet, (iii) cones. The results with excessive light adaptation and the relative asymmetry of *b*-wave and off-effect in the light-adapted eye suggest that there are at least two types of cones overlapping with respect to distribution of sensitivity to spectral light.

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THE QUANTITATIVE ESTIMATION OF HISTAMINE IN THE BLOOD

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Since the discovery of the powerful biological properties of histamine, various methods have been devised for its quantitative estimation. These have been of two general types, chemical and physiological. Chemical methods have not been successfully applied to blood. Methods involving as their final test one or more of the physiological responses to histamine have been used to determine the blood histamine equivalent.

In order to carry out an accurate biological assay of histamine, preliminary treatment of the blood has been necessary. Harris [1927] took blood direct from the vein of human subjects into absolute alcohol, allowed it to stand for some hours, filtered, dried the filtrate *in vacuo*, took up the residue in 0·9 p.c. saline and estimated it directly by the cat's blood pressure. Best & McHenry [1930] determined the histamine content of various tissues by preliminary treatment with hot HCl, removal of acid by drying *in vacuo* and distillation with alcohol, extraction of the residue with water, neutralization, filtration and final estimation of the filtrate on the blood pressure of the etherized cat. Dog's blood treated in this manner was found to contain 0·4 mg. histamine per kg. blood.

Barsoum & Gaddum [1935*a*] have developed a method for estimating the histamine content of blood, involving the preparation of an extract followed by its pharmacological assay. Preparation of the extract requires, briefly, the five following steps:

(1) Precipitation with trichloroacetic acid followed by filtration.

(2) Removal of trichloroacetic acid from the filtrate with ether.

(3) Hydrolysis of the filtrate with HCl with subsequent removal of excess acid by drying *in vacuo* and distillation with alcohol.

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(4) Extraction of the residue with hot alcohol saturated with NaCl, filtration and removal of the alcohol from the filtrate.

(5) The dried residue is taken up in water, neutralized to litmus and is ready for pharmacological assay.

The method as outlined above has been used in various types of physiological experiment [Barsoum & Gaddum, 1935*b*; Anrep & Barsoum, 1935; Anrep *et al.* 1936].

Recently we commenced the routine use of this method in order to follow the changes in the histamine content of dog's blood under certain conditions. All estimations were done in duplicate or triplicate. The process of extraction was time consuming, and inconsistencies occurred in the histamine equivalents of extracts from the same sample of blood, which we felt could not be attributed to variations in technique. Duplicate extracts did not always show discrepancies. In the estimation of twenty different samples of dog's blood from each of which two or three separate extracts were prepared, a variation between the duplicate or triplicate extracts of over 30 p.c. occurred in seven instances. Duplicates of the remaining thirteen samples had an average percentage difference of 6. In comparing known standard solutions of histamine we observed the extreme sensitivity of the lower ileum of the guinea-pig to minute variations in histamine. It was decided therefore to subject the method of extraction to analysis with the hope of shortening the time necessary to prepare the extract and, if possible, increasing the accuracy of the method.

Experimental procedure

The Barsoum & Gaddum [1935*a*] method was first applied to histamine added to water, in quantities comparable to that found in blood. The facts acquired from this analysis were then applied to whole blood and a modified procedure evolved. The histamine equivalents were estimated as a routine on the lower ileum of the guinea-pig. The results were at times checked on the blood pressure of the etherized cat. Finally it was necessary to demonstrate that the substance estimated in the extracts of the modified method was similar pharmacologically to pure histamine. In the routine use of the Barsoum-Gaddum method we followed two suggestions made by Prof. J. H. Gaddum. 10 c.c. of concentrated HCl were added prior to boiling in place of 10 c.c. *N* HCl. Also the alcoholic extraction was carried out at a temperature of 50–52° C., with absolute alcohol saturated with NaCl at that temperature. Apart from these two differences we applied the method as originally published. All estimations are expressed in terms of histamine base.

Estimation of histamine added to water

The occurrence of differences in the histamine equivalents of duplicate samples of blood suggested that a variable amount of histamine was being lost in the extraction process. To determine the loss of histamine in each step of the process, we carried out an analysis of the method with histamine added to water.

During these experiments it was found unnecessary to extract with ether for removal of trichloroacetic acid. Boiling with concentrated HCl destroyed most of the trichloroacetic acid, the remainder being removed by evaporation with hot alcohol. Inconsistent losses of histamine occurred in the final extraction with hot alcohol saturated with NaCl, while extraction with water resulted in complete recovery (Table I).

TABLE I. Recovery of histamine from water

Steps performed	Estimated histamine γ per c.c.	Histamine originally present γ per c.c.	Percentage recovery
Group 1. Trichloroacetic acid, ether, HCl, alcoholic extraction	0·055	0·05	110
	0·035	0·05	70
	0·347	0·5	69
	0·307	0·5	61
Group 2. Trichloroacetic acid, HCl, alcoholic extraction	0·040	0·05	80
	0·034	0·05	68
	0·363	0·5	73
	0·344	0·5	69
Group 3. HCl, alcoholic extraction	0·034	0·05	68
	0·034	0·05	68
	0·032	0·05	64
	0·032	0·05	64
	0·347	0·5	69
	0·363	0·5	72
Group 4. HCl, water extraction	0·050	0·05	100
	0·052	0·05	104
	0·049	0·05	99
	0·500	0·5	100
	0·500	0·5	100

The modified method of extraction

Upon the basis of the above experiments a modified method of extraction has been evolved. It consists of the following steps:

(1) 10 c.c. of blood are added to 15 c.c. of 10 p.c. trichloroacetic acid and after standing for ½–1 hour filtered by suction.

(2) 10 c.c. of concentrated HCl are added to the filtrate and the solution boiled for 90 min. Excess acid is removed by repeated evaporation in the presence of alcohol.

(3) The dried residue is extracted three times with 2 c.c. of water, and the combined extracts are filtered. The filtrate is neutralized and made up to 10 c.c. with water. The extract is then assayed pharmacologically.

Step 1. Filtration by suction must be complete. Cloudy filtrates tend to give high values in the final estimation. The precipitate is washed four times with 5 c.c. trichloroacetic acid.

Step 2. The boiling with concentrated HCl is carried out with a reflux air condenser, water being added to prevent desiccation. Towards the end of the boiling period the volume is reduced to less than 5 c.c., 10 c.c. absolute alcohol are added and the residue dried *in vacuo* on a hot bath.

Step 3. 2 c.c. of water are thoroughly mixed with the dried residue and allowed to stand for a few minutes. Filtration is commenced and the extraction with water repeated until 6 c.c. have been used. The filtrate is neutralized with *N*/5 NaOH. Neutralization to litmus has in our experience been unsatisfactory. Excess alkali may be added without change in the reaction to litmus. We have found the guinea-pig intestine extremely sensitive to slight variations in *p*H. By the use of brom-thymol blue and thymol blue a satisfactory adjustment of *p*H is possible. Brom-thymol blue is used merely to indicate when the correct *p*H is being approximated. The colour given by one drop of thymol blue plus one drop of Tyrode's solution is accurately matched with similar quantities of the indicator and the extract. 1·0–1·5 c.c. of *N*/5 NaOH is generally necessary. To reduce the acid content of extracts from dog's whole blood to within this range usually requires drying three times with alcohol, while with rabbit's whole blood the process must be repeated four to five times. In the neutralization of extracts from whole blood a fine precipitate forms. Its presence does not interfere with the final estimation.

The potassium content of the extracts. It was anticipated that in extracts from whole blood the potassium content might be sufficiently high to interfere with the reaction of the guinea-pig ileum to histamine. We are indebted to Mr R. A. Gregory for carrying out potassium estimations on a series of extracts by the Kramer & Tisdall [1921] method. Extracts from whole blood contained on the average 21 mg. p.c. potassium which is approximately twice that present in Tyrode. Small volumes of the extracts are added to a relatively large quantity of Tyrode's solution. Addition of the largest dose of the extract used in testing to the volume of Tyrode's solution bathing the intestine would elevate the potassium concentration from 10·4 to 12·5 mg. p.c. Raising the potassium content to this and slightly higher levels had no effect upon the intestine, and did not interfere with its contraction in response to histamine.

Total molar concentration of the extracts. Estimates of the total molar concentration of the extracts have been made by the Baldes [1934] modification of the Hill thermoelectric method. We are grateful to Mr G. H. Benham for carrying out these determinations. Early in this research it was found that, in order to obtain a molar concentration similar to that of Tyrode's solution, it was necessary to dilute the extracts to 10 c.c. Adoption of this procedure gave molar concentrations approximately that of Tyrode (Table II).

TABLE II. The total molar concentration of extracts

	Sample	Method of extraction	Total molar concentration expressed as p.c. NaCl
1.	Whole blood	Modified	0·899
2.	"	"	0·950
3.	"	"	0·977
4.	"	"	1·009
5.	"	"	0·875
6.	"	"	0·860
7.	"	"	0·946
8.	"	"	0·884
9.	"	Barsoum-Gaddum	0·883
10.	"	"	1·040
11.	Tyrode's solution	Nil "	0·957

The pharmacological assay of the extracts

The lower portion of the ileum of the guinea-pig has been used for the routine estimation of histamine. Approximately 2 in. of the intestine are suspended in a bath containing 2·5–3 c.c. Tyrode's solution. Titration is carried out according to a definite time schedule. One-half minute is allowed for contraction, one-half minute for washing and relaxation and one-half minute for rest. The unknown solution is alternated with a constant dose of standard histamine solution (Fig. 1). The standard consists of Tyrode's solution containing 0·2γ or 0·1γ histamine per c.c. (Fig. 1). We have observed that such solutions lose approximately 30 p.c. of their activity on standing for 24 hours in a refrigerator. The 1 : 10 million standard has been adopted in the assay of blood containing very little histamine. The intestine is sufficiently sensitive to respond proportionally to small variations in the dose of this dilute standard (Fig. 2). Blood with a histamine equivalent of 0·02γ per c.c. may be accurately estimated.

Following a suggestion by Prof. J. H. Gaddum, atropine has been added to the Tyrode. Concentrations of 1·0–0·5γ per c.c. in the stock solution facilitate the titration by ironing out spontaneous movements and shortening the relaxation time of the gut. The presence of atropine

reduces the sensitivity of the intestine to histamine and may cause difficulty in the estimation of weak extracts.

Fig. 1. Histamine equivalent estimation by the guinea-pig intestine. Doses of extract (*Ex*) and standard histamine solution (*St*) added to 3 c.c. perfusion fluid are indicated below the contractions.

A. Rabbit's blood. 0·16 c.c. extract equivalent to 0·1 c.c. 1 : 5 million standard.
B. Dog's blood. 0·19 c.c. extract equivalent to 0·04 c.c. 1 : 10 million standard.

Blank extracts prepared by passing water through the entire process had a negligible effect on the intestine. Histamine added to these extracts in known amounts gave correct titrations. Histamine added to extracts of blood was also quantitatively estimated. The process of extraction did not produce substances which interfere with the action of histamine.

The recovery of histamine added to blood

In estimating the recovery of histamine added to blood it was considered necessary to commence with blood containing little or no histamine. Advantage was taken of the observation that histamine is rapidly removed from blood by perfusion through the dog's kidney [Best & McHenry, 1930; Steggerda *et al.* 1935]. Forty-five minutes

perfusion of defibrinated dog's blood through the isolated kidneys by the heart-lung preparation reduced the histamine concentration to a level which we have called zero (Table III). Less than 0·01 γ per c.c. may have been present, but extracts prepared by both the Barsoum-Gaddum and the modified methods had little or no effect on the intestine.

Fig. 2. Contraction of the guinea-pig intestine to increasing doses of 1 : 10 million standard histamine solution. The doses in γ histamine added to 2·5 c.c. perfusion fluid are indicated below the contractions.

Histamine was then added in varying concentrations, and extracts prepared by both methods. Careful estimation of these extracts showed that 90 p.c. or more of the added histamine had been recovered by the modified process, while recoveries with the Barsoum-Gaddum method were lower and less consistent (Table III). In addition to the experiments incorporated in Table III, twelve estimations have been carried out in which histamine was added to blood in quantities unknown to the investigator. The modified method yielded consistent recoveries of approximately 90 p.c.

TABLE III. The recovery of histamine added to blood

To reduce the initial concentration of histamine the blood was perfused through the isolated kidneys

Sample no.	Method of extraction	Estimated histamine γ per c.c.	Added histamine γ per c.c.	Percentage recovery
1, 2, 3, 4	Modified	0·000	0·000	—
5, 6, 7, 8	Barsoum-Gaddum	0·000	0·000	—
9	Modified	0·061	0·066	92
10	,,	0·062	0·066	94
11	,,	0·061	0·066	92
12	Barsoum-Gaddum	0·045	0·066	70
13	,,	0·043	0·066	65
14	,,	0·050	0·066	76
15	Modified	0·091	0·1	91
16	,,	0·094	0·1	94
17	,,	0·095	0·1	95
18	Barsoum-Gaddum	0·050	0·1	50
19	,,	0·050	0·1	50
20	,,	0·062	0·1	62
21	Modified	1·032	1·0	103
22	,,	1·000	1·0	100
23	,,	0·909	1·0	90
25	Barsoum-Gaddum	0·727	1·0	72
26	Modified	9·303	10·0	93
27	,,	9·526	10·0	95
28	Barsoum-Gaddum	6·545	10·0	65
29	,,	6·261	10·0	62

The histamine equivalent of blood to which no histamine has been added

The modified method has been applied to the routine estimation of dog's and rabbit's blood. Blood has been withdrawn under ether anæsthesia and immediately defibrinated. By using a proportional quantity of the various reagents, satisfactory extracts have frequently been prepared from 5 c.c. of blood. The average histamine equivalent of fourteen estimations on blood from five different dogs was 0·032γ histamine per c.c. blood, while that from seven different rabbits was 1·85γ per c.c. blood (Table IV). The agreement between duplicates was within 10 p.c. There was, however, considerable variation between the individual animals of each group.

Pharmacological evidence that the active principle of the extract is histamine

Modification of the method of extraction made it essential to test the extracts by methods other than the guinea-pig intestine. Rabbit's blood has been used throughout this series of experiments, because of its

comparatively high histamine equivalent. The small amount of histamine estimated to be present even in these extracts limits the number of tests which may be carried out.

The histamine equivalent of blood from five rabbits was estimated first by the blood pressure of the etherized cat, and then by the guinea-pig intestine (Table IV). In accord with the observation of Barsoum & Gaddum [1935*a*], atropinization of the cat considerably reduced the histamine equivalent of the extracts (Fig. 3). Following atropinization the values remained slightly higher than those derived from the guinea-pig intestine. With the exception of one experiment, the difference in the histamine equivalents of the two methods did not exceed 10 p.c.

Table IV. The histamine equivalent of rabbit's blood estimated on the guinea-pig intestine and the cat's blood pressure

		Histamine equivalent in γ per c.c. blood		
			Cat's blood pressure	
Exp.	Sample	Guinea-pig intestine	After atropine	Before atropine
18	1	1·60	1·72	2·66
	2	1·66	1·81	2·85
19	1	1·33	1·43	2·00
	2	1·33	1·43	2·00
20	1	0·59	0·62	—
	2	0·63	0·62	—
21	1	2·50	3·12	—
	2	2·66	3·33	—
22	1	2·50	2·66	—
	2	2·50	—	—
23	1	2·15	—	—
	2	2·00	—	—
24	1	2·42	—	—
	2	2·22	—	—
	Average	1·85		

Large doses of the extracts, producing a marked fall in the blood pressure of the cat, had no effect on the blood pressure of the etherized rabbit. The extracts caused a contraction of the uterus of the virgin guinea-pig, similar in character to that produced by histamine.

Large doses of histamine, allowed to act for a prolonged period, greatly reduce or abolish the sensitivity of the guinea-pig intestine to histamine [Feldberg & Schilf, 1930; Barsoum & Gaddum, 1935*a*]. 50–100 γ of histamine in contact with the intestine for 15–30 min. completely abolished its response to the standard histamine solution and the extracts, while that to barium chloride was only slightly diminished.

Doses of this magnitude could not be delivered by the extracts. Large quantities of the extracts allowed to act for ½–1 hour greatly reduced, although they did not completely abolish, the response to the extracts and to the standard histamine solution.

Following the technique of Koessler & Lewis [1927], we have tested the effect of the extracts on the bronchial musculature of the guinea-pig.

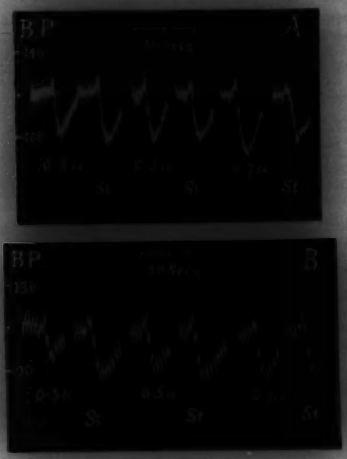

Fig. 3

Fig. 4

Fig. 3. The histamine equivalent of an extract from rabbit's blood estimated by the cat's blood pressure, A before atropinization and B after atropinization. *St* is response to 1γ histamine standard. Doses of extract injected indicated in c.c. Before atropinization 0·5 c.c. extract equivalent to 1γ histamine, after atropinization 0·7 c.c. extract equivalent to 1γ histamine.

Fig. 4. Respiratory pulmonary excursions of the decerebrate guinea-pig, recorded by means of a perforated needle placed in the pleural cavity and connected to a bellows recorder. At arrow, 1 c.c. extract of rabbit's blood injected intravenously. Pulmonary excursions cease.

Intravenous administration of the extract produced responses exactly similar to pure histamine (Fig. 4). Large doses of the extracts caused death of the guinea-pig, and at autopsy the voluminous lungs typical of histamine overdose were found [Dale & Laidlaw, 1910].

Discussion

The chief modifications in the preparation of the extract have been the omission of the unnecessary removal of trichloroacetic acid with ether and the substitution of final extraction with water in place of alcohol. Our results indicated that repeated extraction with hot absolute alcohol saturated with NaCl did not remove all the histamine present. By extracting three times with moderate amounts of dilute alcohol, Best & McHenry [1931] report approximately 95 p.c. recovery of histamine. Hot 95 p.c. alcohol saturated with NaCl did not increase the recovery of histamine in our experiments. Hanke & Koessler [1920] used preliminary hot alcoholic extraction, thereby dividing their material into alcohol extract and alcohol insoluble residue. In applying their method to human blood serum, they added 5·0 mg. histamine to 75 c.c. serum, and recovered 3·5 mg. in the alcohol extract and 0·3 mg. in the alcohol insoluble residue. The remainder 1·2 mg. they believed had been absorbed by the charred material formed in acid hydrolysis and had not been removed by their final alkali-amyl-alcohol extraction. In the isolation of histamine from liver and lung [Best *et al.* 1927] and from spleen [Dale & Dudley, 1929], the most serious loss of histamine occurred in the final alkaline extraction with absolute alcohol. In our experiments, extraction with alcohol did not completely remove the histamine from the dried acid residue. Substitution of aqueous extraction gave higher and more consistent recoveries.

The histamine equivalents of blood from normal dogs and rabbits have in our experiments been consistently lower than the values reported by Barsoum & Gaddum [1935*a*]. The difference cannot be attributed to the adoption of the modified procedure. Using the Barsoum-Gaddum method we obtained an average value of 0·026 γ per c.c. blood from eleven estimations on the blood of five dogs. A histamine equivalent of 2·6 γ per c.c. is the highest value we have yet obtained from whole rabbits' blood.

The effect of active substances other than histamine, which may be present in the extracts, has as far as possible been eliminated. When estimated by the cat's blood pressure, atropinization definitely reduced the histamine equivalent values (Table IV). The effect of the extracts on the blood pressure of the unatropinized cat is, as Barsoum & Gaddum [1935*a*] have stated, probably partly due to choline. When determining the histamine equivalent by the guinea-pig intestine, we have added atropine to the stock Tyrode solution.

The pharmacological evidence indicates that the active principle estimated in the extract is histamine. The chemical proof that histamine is present in the blood is, however, still lacking.

Summary

1. The Barsoum-Gaddum method of extracting histamine from blood has been investigated.

2. A shorter method of extraction has been developed.

3. Application of this method to whole blood yields consistent histamine equivalent values.

4. Pharmacological evidence is submitted in support of the view that the active principle of the extracts is histamine.

I wish to express my sincere thanks to Prof. C. Lovatt Evans and Prof. J. H. Gaddum for their help and advice throughout this investigation. Part of the expense of this research was defrayed by a grant from the Donald C. Balfour Educational Fund.

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THE ABSORPTION OF AMINO ACIDS AND THEIR DISTRIBUTION IN THE BODY FLUIDS

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The problem of the absorption, distribution and utilization of amino acids in the body is of fundamental importance, yet our knowledge of it is still very incomplete. It is now well recognized that the digested proteins pass through the epithelial cells of the mucous membrane of the small intestine as amino acids (although it has recently been suggested that some portion may also penetrate as small peptides), and the experimental evidence for this view was well summarized by Van Slyke & Meyer [1913] in the introduction to one of their important series of papers dealing with amino-acid metabolism. In the present paper, however, we are only concerned with the absorption of these substances after they have penetrated the epithelial layers and reached the tissue spaces of the villi.

This communication deals with an experimental investigation into two problems, which are interrelated to such a degree that they can hardly be investigated separately. These problems are:

(1) The absorption of amino acids from the tissue spaces of the intestinal villi by the blood capillaries and lymph lacteals.

(2) The distribution of the amino acids in the blood after their absorption following protein digestion, and also their distribution in the states of fasting and starvation.

Briefly summarized, the present knowledge on these questions is as follows:

(1) After their passage through the superficial epithelium into the tissue spaces of the villi, it is commonly believed that amino acids, like glucose and other crystalloids, are removed by way of the capillaries of the portal venous system, and that the fats are almost entirely removed

by the lymph lacteals. This view implies a selective activity on the part of the endothelium lining these vessels, though all the observations made in recent years on the capillary wall in lymph and œdema formation have shown that it is unnecessary to invoke any other than physical and mechanical factors for their explanation.

The only observations concerned with the relative importance of the blood and lymph in the removal of amino acids from the villous tissue spaces are those of Hendrix & Sweet [1917]. These authors estimated the concentrations of the amino acids in the blood of the external jugular vein and the lymph from the thoracic duct of four dogs after introducing either peptone, milk or amino acids into their intestines. They found that the level of the amino-acid nitrogen in the lymph rose to a higher level than that in the systemic blood, although in the previously fasting animals the reverse had been the case. These experiments lose much of their force because the blood from the external jugular vein does not represent that from the intestinal villi.

(2) To Van Slyke & Meyer [1913] belongs the credit for first estimating quantitatively the concentrations of the amino acids in the blood and tissues. They demonstrated that amino acids are rapidly taken up by the tissues from the blood without suffering any intermediate change. There is a definite upper limit to the absorption of amino acids by skeletal muscle, but some of the viscera are more elastic in this respect, and their content of amino acids may attain twice the concentration found in the muscles. The amino acids never completely disappear from the blood, a state of equilibrium developing between the tissues and the blood. The amino acids taken up by the liver rapidly disappear, so that, while in the early stages of absorption their concentration in it may be virtually doubled, 2 or 3 hours later it may have fallen again to its usual post-absorptive level; the concentration of amino acids in the muscles shows no corresponding fall during this time. This disappearance of amino acids from the liver is accompanied by an increase in the blood urea, most marked in the hepatic veins, showing that the liver desaturates itself of amino acids by metabolizing them, and hence maintains indefinitely its power of removing them from the circulating blood.

Van Slyke & Meyer [1913] also showed that considerable concentrations of amino acids are present in the tissues during starvation, so that they appear to be intermediate substances in the breaking down of body proteins, as well as in their synthesis.

The blood passing from the different organs should show variations in its amino-acid content in accordance with the changes in activity of

such organs: but we can find no reference to any systematic examination of the blood from different parts of the body having been made simultaneously under these various changes in activity.

The experiments recorded in this paper will be described under the following headings:

(1) Local variations in the amino-acid concentration in the blood and plasma flowing in certain vessels during (i) fasting, (ii) starvation, (iii) feeding with peptone.

(2) The interrelation of plasma and lymph amino-acid concentrations during (i) fasting, (ii) starvation, (iii) feeding with peptone.

(3) Amino-acid concentrations in the blood, plasma and lymph after feeding with other proteins.

Methods

The animals used were cats, which were fed on milk up to the time of the commencement of the experiment. Samples of blood were taken from the following vessels: (*a*) carotid artery as an index of the concentration of amino acids at the arterial ends of the capillaries; (*b*) inferior vena cava as that of the venous blood coming from the somatic tissues generally; (*c*) superior mesenteric vein as that coming from the small intestines; (*d*) hepatic vein; and samples of lymph either from the thoracic duct or from the cisterna chyli.

About 6 c.c. of blood and about 1–2 c.c. of lymph were obtained from the vessels sampled. When lymph was obtained from the thoracic duct, a variable time was required to make the preparation and collect the lymph. In those experiments where this procedure was adopted it constituted the preliminary part of the operation, so that the minimum of time was spent in procuring the blood samples immediately afterwards. The method used in preparing the thoracic duct of the cat for the collection of lymph has already been described [Bolton, 1921].

The following describes the order and method of sampling the blood in the different vessels. First, blood was obtained from the carotid artery by the usual method of introducing a cannula into the vessel. The abdomen was then opened and the cisterna chyli exposed by reflecting the left kidney, the intestines being carefully protected with gauze well wrung out of warm saline solution. A little pressure on the loops of small intestines sufficed to fill the cisterna if it were lax. A hypodermic needle was introduced into the cisterna and the chyle gently sucked out. The organs were then replaced in position, and the splenic vein was exposed at its junction with the superior mesenteric vein. A fine cannula was

introduced down the splenic vein until its end was flush with the mesenteric vein; blood was then withdrawn into a glass syringe. It was found advisable to ligature the splenic artery to avoid swelling of the spleen. Blood was next obtained from the inferior vena cava. The small intestines were turned over to the left, and a cannula was introduced down the right renal vein until its end was flush with the vena cava; blood was then withdrawn into a syringe. Finally the hepatic vein was sampled. In the cat the inferior vena cava enters the lower border of the right lobe of the liver and passes through its substance, receiving in its progress several moderately large veins coming from the liver, and finally the main hepatic vein as it pierces the diaphragm. After obtaining the blood from the inferior vena cava, that vessel was ligatured as it entered the liver, and a few moments were allowed to elapse for the vena cava proximal to the ligature to become filled with purely hepatic blood. The chest was then rapidly opened, and the inferior vena cava was emptied upwards and clamped at its entrance into the right auricle. On puncturing the vena cava, which thus was only receiving hepatic blood, the latter soon partially filled the right pleural cavity, and was withdrawn from it with a syringe. A small quantity of heparin was placed in all the collecting tubes to prevent clotting.

The time between the collection in the tubes of the superior mesenteric venous blood and the chyle was certainly no longer than 10 min. In addition, as a check against the occurrence of any marked change in the general level of the blood amino acids during the course of the operation, in some experiments a second sample was taken, near the end of the operation, from the carotid artery for comparison with the first sample from the same vessel. No material differences between the two estimations were found. The whole operation of obtaining these samples was carried out as rapidly as possible under ether anæsthesia; it usually took about half an hour from the collection of the first arterial sample to the final hepatic one.

A small quantity of blood was retained for analysis, and the remainder rapidly centrifuged to separate the cells and plasma. Amino-acid estimations on the whole blood, the separated plasma and the lymph were made by the method of Folin [1922], after precipitation of the proteins by tungstic acid. In this way information was obtained both as to the distribution of the amino acids in the blood of different vessels, and as to the concentrations in the plasma with which the tissues and tissue fluids are in close contact.

Results.

(1) *Local variations in the amino-acid concentrations in the blood and plasma flowing in certain vessels during* (i) *fasting,* (ii) *starvation,* (iii) *feeding with peptone*

A. *Blood*

(i) *Fasting state.* An attempt was first made to determine the condition which was as nearly one of equilibrium as possible, i.e. a basal state in which all the amino acids resulting from the absorption of food have been disposed of, and in which the signs of starvation have not yet appeared. The term "fasting" was applied to animals which had been deprived of food for between 24 and 48 hours, and which may be regarded as having been in this essentially basal state.

The results obtained during fasting are set out in Table I. From this table it can be seen that the average amino-acid concentrations in the venous blood samples, with the exception of the hepatic venous blood,

Table I. The concentration of the amino acids in the whole blood in various vessels during fasting.

	Amino-acid nitrogen (mg. p.c.)			
Cat no.	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava
105	8·6	9·2	7·7	8·1
106	5·8	6·1	4·8	6·0
108	6·6	6·3	5·5	6·2
109	6·1	7·8	7·0	7·6
Averages	6·8	7·4	6·3	7·0

are slightly higher than in the arterial blood. It appears, therefore, that additions of amino acids to the blood are taking place both from the intestinal tissues drained by the superior mesenteric vein, and from the somatic tissues whose venous blood passes into the inferior vena cava. Since the amino-acid concentration in the superior mesenteric vein is slightly, but uniformly, higher than that in the inferior vena cava, the final stages of absorption of protein digestion products from the small intestines are probably not entirely at an end. In the state of fasting, however, the relative slowness of the circulation rate through the portal system, in comparison with that occurring in digestion, tends to give an exaggerated idea of the actual quantity of amino acids exchanged between the intestines and the blood flowing through them.

The concentration of the amino acids in the hepatic venous blood is much lower than that in either the superior mesenteric vein or the

inferior vena cava; in fact, in three of the four cats examined it was also below that found in the arterial blood. The extremes of difference lie between the superior mesenteric and hepatic venous blood: the actual reduction in concentration during passage through the liver having a mean value of 1·1 mg. amino-acid nitrogen per 100 c.c. of blood. It is thus clear that the active removal of amino acids from the blood by the liver continues to take place even in the condition of fasting.

Taken as a whole, the general level of the blood amino acids is lower during fasting than in either of the two other states of starvation and feeding which have been examined.

(ii) *Starvation state.* The term "starvation" was applied when the cats had been deprived of food for more than 48 hours and less than 168 hours. The blood analyses obtained during this group of experiments are set out in Table II. This table shows that the average concentration

TABLE II. The concentration of the amino acids in the whole blood in various vessels during starvation.

		Amino-acid nitrogen (mg. p.c.)			
Cat no.	Hours of starvation	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava
110	72	8·0	8·8	7·9	9·5
111	72	8·7	8·4	7·4	8·3
112	96	7·9	8·1	7·7	8·6
113	120	7·9	7·7	7·1	8·1
115	168	7·8	7·6	6·8	7·6
Averages (all)		8·1	8·1	7·4	8·4
(last four)		8·1	8·0	7·3	8·2

of the amino acids in the blood in the superior mesenteric vein is practically the same as that in the artery, whereas that in the blood in the inferior vena cava has risen above the level of both. This indicates that when the state of starvation has set in, absorption from the intestines has come to an end, and absorption from the somatic tissues forms the principal source of amino acids for the body generally.

The concentration of amino acids in the hepatic venous blood is again below those found in the remainder of the vessels sampled, but it is higher in relation to that in the blood of the superior mesenteric vein than during fasting, the actual difference having a mean value of 0·7 mg. amino-acid nitrogen per 100 c.c. of blood. It appears, therefore, that there is an even less active removal of amino acids by the liver in the state of starvation than during fasting.

Taken as a whole, the general level of the amino-acid concentrations in the various vessels examined is distinctly higher than in the fasting

state. This has taken place in spite of the fact that in all the cats examined there were still considerable reserves of adipose tissue, so that the raised concentration of amino acids does not represent the beginning of the "pre-mortal rise" observed by Marino [1923].

(iii) *Peptone feeding.* Peptone was administered in amounts varying from 5 to 20 g. in 50 c.c. of water. Under these conditions absorption of amino acids from the lumen of the intestines must be proceeding at nearly, if not quite at, its maximal rate.

In the experiments about to be described the peptone solution was given by stomach tube usually 4–5 hours before the samples of blood and lymph were obtained.

The results of these experiments are given in Table III. A striking phenomenon observed was the great rise in the concentration of amino acids in the blood samples obtained from all parts of the vascular system

TABLE III. The concentration of the amino acids in the whole blood in various vessels during peptone digestion.

			Amino-acid nitrogen (mg. p.c.)			
Cat no.	Grams of peptone	Hours after feeding	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava
22	20	4	16·5	20·9	18·7	17·1
25	20	5	17·4	21·2	16·8	15·8
26	20	5	17·7	20·9	16·2	16·8
27	20	5	13·9	17·7	14·1	14·1
28	20	5	16·7	20·3	18·0	16·2
36	20	4	—	21·1	17·4	—
37	20	5	—	19·1	15·3	—
Averages (all)			—	20·2	16·6	—
(first five)			16·4	20·2	16·6	16·0
21	10	5	12·8	16·1	13·9	12·5
43	10	4	—	13·6	—	—
44	10	4	—	21·1	—	—
Average				16·9		

examined. Most notable, as might be expected, was the rise that took place in the blood of the superior mesenteric vein, which indicated the exceedingly active absorption from the lumen of the intestines. The mean difference between the concentrations in the arterial and superior mesenteric venous blood was nearly 4 mg. amino-acid nitrogen per 100 c.c. of blood, and comparatively little variation was found among any of the five cats examined.

This very large absorption is nearly balanced by the great removal of amino acids as the blood passes through the liver; the cumulative effect, however, of the slight difference between the hepatic venous blood and

the arterial blood gradually raises the amino-acid concentration throughout the whole vascular system. This removal results, as Van Slyke & Meyer [1913] have shown, partly from their local destruction and partly from their local storage. The blood in the inferior vena cava had, on average, an amino-acid nitrogen content of 0·4 mg. below that of the arterial blood, showing that a similar, though much less active, removal of amino acids took place during the passage of the blood through the muscular regions drained by that vein. Neither this removal, however, nor the activity of the liver prevented the amino-acid concentration in the peripheral veins from rising to nearly double the values found either in fasting or starvation. A similar rise to nearly twice the initial fasting value was found by Folin & Berglund [1922] in the peripheral venous blood of a subject 3 hours after taking by mouth a large quantity of gelatin solution, and Witts [1929] has described a comparable rise after the administration by mouth of 50 g. of glycine to a normal person.

B. *Plasma.*

(i) *Fasting state.* The results of the plasma analyses are set out in Table IV. It will be seen that the amino-acid concentrations in the plasma in the various vessels sampled follows the same order as that of

Table IV. The concentration of the amino acids in the plasma in various vessels and the lymph during fasting.

		Amino-acid nitrogen (mg. p.c.)			
Cat no.	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava	Lymph
7	5·5	6·7	5·2	—	7·3*
9	8·4	10·5	7·7	—	9·3*
10	7·6	9·1	7·4	—	8·1*
Averages	7·2	8·8	6·8	—	8·2*
105	5·6	6·2	5·5	5·8	7·0†
106	4·8	5·0	4·4	5·1	5·8†
108	5·5	5·5	4·9	5·5	5·9†
109	3·9	4·6	4·5	4·8	4·7†
Averages	5·0	5·3	4·8	5·3	5·9†

* Thoracic duct lymph. † Cisterna chyli lymph.

the corresponding bloods. The relative proportion of the amino-acid concentration in the plasma to that in the blood is the same in the artery and the superior mesenteric vein, but it is higher in the hepatic vein and inferior vena cava. This indicates a rather lower concentration in the red corpuscles in these latter vessels. The problem of the movements of

amino acids between plasma and corpuscles will not be taken up in the present paper.

(ii) *Starvation state.* The results of the analyses are shown in Table V. In this state the concentrations in the plasmas again fall into the same order as those of the bloods from which they were obtained, but, while

TABLE V. The concentration of the amino acids in the plasma in various vessels and the lymph during starvation.

		Amino-acid nitrogen (mg. p.c.)				
Cat no.	Hours of starvation	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava	Lymph
111	72	5·4	5·3	4·7	5·3	6·3*
112	96	4·8	5·5	5·1	6·4	6·8*
113	120	5·1	5·1	4·8	5·6	5·7*
114	168	4·6	4·3	3·6	5·2	6·0*
115	168	4·5	4·5	3·8	4·7	5·7*
Averages		4·9	4·9	4·4	5·4	6·1*

* Cisterna chyli lymph.

the average plasma amino-acid concentration is approximately the same as that previously found in the fasting state, the average blood concentration, and consequently the corpuscular concentration, has risen considerably. The amino-acid concentration in the blood of the inferior vena cava has risen considerably, though it shows the lowest relative corpuscular concentration. It is clear, however, from these observations that in the state of starvation the distribution of amino acids in the blood is more in favour of the corpuscles than the plasma.

(iii) *Peptone feeding.* The results of the analyses are shown in Table VI. After feeding with a food which releases rapidly very large quantities of amino acids in the intestines, the concentrations of these substances follow the same order in the plasmas and bloods of the various vessels examined. If, however, the relative concentrations of the amino acids in the plasmas and their corresponding bloods be compared, it is found that after peptone feeding they are higher than in both starvation (about 50 p.c.) and fasting (about 25 p.c.). During the rapid absorption of the amino acids liberated by peptone digestion, therefore, the plasma contains relatively larger concentrations than the corpuscles.

(2) *The interrelation of plasma and lymph amino-acid concentrations during* (i) *fasting,* (ii) *starvation,* (iii) *feeding with peptone*

The tissue fluid filling all the intercellular spaces is the medium into which the cells of the part discharge their waste products, and derive the oxygen and other substances necessary for their metabolism. This

TABLE VI. The concentration of the amino acids in the plasma in various vessels and the lymph during peptone digestion.

			Amino-acid nitrogen (mg. p.c.)				
Cat no.	Grams of peptone	Hours after feeding	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava	Lymph
22	20	4	16·9	23·5	19·6	16·0	18·9*
25	20	5	16·1	21·5	16·5	16·5	20·6*
26	20	5	14·7	18·1	15·0	14·7	17·1*
27	20	5	12·9	15·1	11·3	12·2	13·4*
28	20	5	15·5	19·1	17·2	15·0	15·6*
	Averages		15·2	19·5	15·9	14·9	17·1*
36	20	4	—	22·0	16·7	—	18·6†
37	20	5	—	20·7	14·3	—	16·2†
39	20	4	—	23·2	—	—	20·7†
120	20	4	9·6	13·4	9·3	—	11·7†
43	10	4	—	15·0	—	—	12·3†
44	10	4	—	22·1	—	—	18·5†
51	5	4	—	16·1	—	—	14·3†

* Thoracic duct lymph. † Cisterna chyli lymph.

fluid is constantly renewed from the capillaries, and is altered in composition by the metabolism of the tissues before being carried away by the lymphatics or reabsorbed into the capillaries themselves. The lymph obtained from the lymphatics therefore represents that portion of the tissue fluid which has escaped reabsorption by the blood vessels. It is thus clear that the blood entering the part by the arterial capillaries must have a slightly different composition from that leaving it by the venous capillaries. Two classes of substances pass through the capillary walls—water and crystalloids; and the inward passage, or absorption, of these substances takes place through the venous capillaries since their hydrostatic pressure is a little the lower. The rapid flow of blood through the part carries away these absorbed substances, so that the composition of the blood traversing it is continually maintained at the level of that of the general systemic blood. The composition of the lymph, moreover, is closer to that of the transuded fluid the greater the rate at which the latter is produced, for it is then less modified by the local tissue metabolism.

The condition of the tissue fluid of the villi differs from that in other parts of the body because, unlike them, it is periodically flooded with water and crystalloids entering through the intestinal mucous membrane. There are thus two aspects to the problem of absorption of tissue fluids from the intestinal villi: (i) that during the condition of fasting, when the formation and reabsorption of the tissue fluid resembles that occurring

in other parts of the body, (ii) that during the rapid absorption of foodstuffs, when the tissue fluid of the villi is further altered by the passage of these substances into it from the lumen of the intestines. The lymph collected from the mesenteric trunks or cisterna chyli in the second of these conditions is, probably, as regards its amino-acid concentration, almost entirely representative of the tissue fluid of the villi only; and thus indicates the change in the composition of the tissue fluid which is due to absorption from the lumen of the intestines rather than that due to transudation from the capillaries. We believe, therefore, that the lymph obtained from the cisterna chyli during digestion is chiefly representative of the tissue fluid of the villi, but that the lymph obtained during fasting or starvation represents the tissue fluid of the general structures of the intestines as a whole, since, as Starling [1894] showed, the lymph draining into the thoracic duct of the resting animal is derived almost entirely from the intestines.

That the lymph in the cisterna chyli represents the tissue fluid of the intestine seems probable from the following considerations. It is improbable that the composition of the lymph can be greatly altered in its rapid passage down the lacteals. The pressure gradient from the tissue spaces, into which the lymph is put out from the capillaries fairly rapidly owing to their relatively high permeability, aided by the pumping action of the muscles in the intestinal wall, the contractions of the villi and the oscillations of pressure in the cisterna chyli accompanying respiration, suffices to cause a fairly constant and rapid flow. The terminal lymphatic plexus in the tissues is a closed system of tubes, so that it might be argued that, during its passage from the tissue spaces into the lymph capillaries, the lymph was changed in composition. It appears to us that such an argument loses sight of the high degree of permeability of the lymph capillaries. Their endothelial walls are readily permeable to small solid particles, and therefore one is driven to the conclusion that the proteins and other soluble constituents of the tissue fluids pass easily through the wall owing to the hydrostatic pressure in the tissue spaces. This pressure is maintained by the constant passage of fluid through the blood capillary walls, and although it does not approach the hydrostatic pressure in the blood capillaries responsible for its output, the pressure of the fluid causes turgescence in the tissue spaces, a turgescence further increased by the muscular contraction of the villi. The tissue fluid, owing to this turgescence, quite easily oozes through the walls of the lymph capillaries, and we believe, therefore, that the lymph in the cisterna chyli corresponds quite closely with the fluid in the tissue spaces from which it

has come. These tissue spaces are partly in the villi and partly in the other parts of the intestinal wall and, in the states of fasting or starvation, the lymph represents a mixture of the contents of both kinds of space, as we have already stated. After feeding peptone, however, the rapid absorption of amino acids by the villi impresses the newly developed characteristics of the villous fluid upon the lymph, so that in amino-acid concentration it accurately represents the tissue fluid of the villi as contrasted with that of the intestinal wall as a whole.

The problem under review in this part of the paper is to determine whether or not the concentrations of the amino acids in the plasma and lymph in the various states of fasting, starvation and feeding with peptone, are compatible with these substances being freely diffusible into both lymphatics and blood capillaries, and therefore that their absorption is in accordance with physical laws, so that it is superfluous to assume vital activity on the part of the capillary endothelium.

The results of the estimations of amino-acid concentrations in the cisterna lymph and the thoracic duct lymph are given in Tables IV, V and VI. The figures for both types of lymph should be compared with those of the plasma, not with whole blood, because the interchange occurs through the capillary endothelium between the plasma and the tissue fluid.

The three fluids to be considered in relation to absorption from the villi are the plasma from the arterial capillaries bringing blood to the intestines, the plasma of the venous capillaries carrying blood away from them, and the cisterna lymph as representing the tissue fluid of the villi.

In certain experiments we have utilized thoracic duct lymph. After peptone feeding the estimations of the thoracic duct lymph show that its content of amino acids has the same relation to the superior mesenteric plasma as the cisterna lymph has, and that in feeding, therefore, lymph from either source would answer our purpose; the amino-acid concentration in the small quantity of lymph from other sources being masked by that in the lymph from the villi. In the state of fasting, in which all absorption from the alimentary tract has not quite ended and starvation has not begun, the thoracic duct lymph is not, however, reliable, as it shows in our experiments a lower relative reading than does the cisterna lymph. Although the thoracic duct lymph obtained during fasting agrees closely with the cisterna lymph, it is a little altered in character by lymph of lower amino-acid concentration derived from other sources. We have, therefore, used the cisterna lymph estimations throughout for comparisons with those of the plasma.

A reference to the tables in question will show that, in the conditions of fasting and starvation, the concentration of amino acids in the cisterna lymph is greater than in the plasma from whatever source the latter is obtained: hence diffusion from the villus tissue fluids to the blood in these two conditions is easily explained. In fasting, when all the fluids are

nearly in equilibrium as regards amino acids, the cisterna lymph has a slight preponderance, which becomes increased as starvation supervenes. In peptone feeding the concentration of amino acids in the cisterna lymph still remains greater than all the venous plasmas except that from the superior mesenteric vein, that is to say, the efferent plasma from the villi. It is greater than that in the afferent arterial capillary plasma, but less than that in the efferent venous capillary plasma.

The explanation of this difference between fasting, starvation and peptone feeding is, we believe, to be found in the sources of the amino acids. In fasting, to a limited extent, and in starvation more particularly, they are solely derived from the tissues themselves. The tissues of the villi and the other structures of the intestinal wall like those of other tissues release amino acids into the tissue fluid round them, whence they diffuse through the capillary walls into the circulating blood plasma. The concentration of these substances in the tissue fluid is higher than that in the locally circulating plasma, and so diffusion into the capillaries occurs: this is our explanation of these findings. In peptone feeding, however, amino acids flood the tissue spaces of the villi after penetrating the epithelial layer, and pass inwards from the periphery towards the centre of the villus. In this state of digestion the percentage of amino acids in the cisterna lymph is intermediate between that of the afferent and that of the efferent plasma, i.e. of the superior mesenteric artery and vein. It is clear that for diffusion into the venous capillaries to occur the concentration of amino acids in the tissue fluids bathing them must be higher than that of the plasma inside them; and yet, if we accept the view that cisterna lymph represents the tissue fluid of the villi, the concentration in this fluid is found experimentally to be lower than that in the plasma.

We believe that the explanation lies in the probability that the concentration of amino acids is greater in the tissue fluids at the periphery of the villus than in its more central parts. It is quite likely that it should be so, and if we accept this hypothesis we have a complete explanation of our experimental results. The arterial and venous capillaries are situated at the periphery of the villus, where they form a close superficial network immediately under the epithelium covering the villus. They enclose the villus in a "mantle", as Mall [1887] described it (see Fig. 1). Through this peripheral capillary network there is a very rapid inflow of blood during the vaso-dilatation accompanying digestion, and the concentration of amino acids in the afferent plasma is the lowest of the three fluids we are discussing. The periphery of the villus, where these capillaries

are situated, is flooded with amino acids passing rapidly in from the lumen of the intestines. The great difference between the concentrations of amino acids inside and outside the capillary walls results in a constant rapid diffusion into this peripheral capillary network, whose blood is continually passing on into the veins and being renewed from the arteries. This rapid removal of the amino acids by the blood lowers their concentration in the tissue fluid locally, but another factor is added. Lymph, with the low amino-acid concentration characterising the afferent arterial plasma, is constantly flowing out of the arterial capillaries towards the lacteals. This lymph flowing away in the lacteals should have the same amino-acid concentration as the arterial plasma from which it comes, but we find it to be greater, and explain this as being due to diffusion of amino acids directly into the central lacteal of the villus, though its concentration there never reaches the height found in the efferent venous plasma. The fact is that diffusion of amino acids under such conditions, occurs both into blood capillaries and lymphatics according to physical laws, but the blood is putting out from the afferent arterial capillaries a lymph which is weaker in amino acids than the tissue fluid of the villi, while at the same time it is absorbing amino acids, especially at the periphery, and removing them at a great rate in the efferent venous capillaries. The result is that the lymph passing away has a higher concentration in amino acids than the arterial plasma, but a lower one than that of the superior mesenteric venous plasma, since the concentration of amino acids in the tissue fluid near their surface of entrance, that is to say, at the periphery round the capillary mantle, is higher than it is in the vicinity of the central efferent lymphatic channels. Absorption no doubt occurs directly into those capillaries which lie immediately in contact with the absorbing epithelium. Absorption occurs almost entirely at the periphery when moderate amounts of water are absorbed, the greater part of this fluid passing into the capillaries and only a little reaching the lacteal in the centre, as Heidenhain [1888] pointed out.

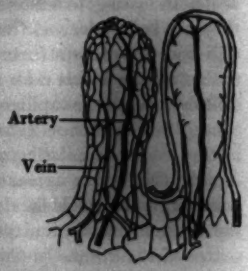

Fig. 1. Tracing from Mall [1887] to show the peripheral arrangement of the capillaries in the villus.

Absorption of amino acids into the blood would not continue at this rate were it not for the fact that the liver is rapidly lowering the amino-acid

concentration of the portal blood as it passes through it to be emptied into the general blood stream. The arterial blood is thus kept from rising to the height of the portal blood, with consequently the continued absorption of amino acids by the capillaries of the villi.

Whilst this absorption is going on in the villi, the concentration of amino acids in the general blood plasma undergoes a slow gradual rise. Under these conditions amino acids are removed from the arterial blood by the tissues generally as it passes through them, so that the concentration of amino acids in the venous blood is lowered, as can be seen by comparison of the arterial with the inferior vena caval blood.

(3) *The amino-acid concentrations in the blood, plasma and lymph after feeding with other proteins*

We now turn to the absorption of amino acids formed in the intestines from the digestion of proteins which are present in natural foods.

(i) *Fat-free proteins.* The figures obtained by feeding with proteins other than peptone show variations in the relationship between the concentrations in lymph and plasma according to the kind of foodstuff administered. During their conversion into amino acids before absorption these substances have to be digested, and the length of time occupied by this process varies according to the composition of the foodstuff. The rate at which they leave the stomach and are supplied to the intestine must also be taken into account in any attempt to estimate the rate at which amino acids, and consequently the amount at any one time, are supplied to the intestinal mucous membrane for absorption. If our hypothesis as regards the absorption of amino acids derived from peptone

TABLE VII. The concentration of the amino acids in the whole blood, plasma and lymph during digestion of "fat-free" proteins.

			Amino-acid nitrogen (mg. p.c.)		
			Superior mesenteric vein		
Cat no.	Quantity and nature of food	Time in hours	Blood	Plasma	Lymph
69	50 c.c. egg-white	4	12·5	10·7	11·2*
70	75 c.c. egg-white	2	10·9	10·3	10·4*
71	90 g. lobster	4	18·5	18·9	15·5*
72	60 g. cockles	5	14·3	12·6	11·7*
73	60 g. haddock	5	17·2	18·7	15·9*

* Cisterna chyli lymph.

(that is to say, in the condition in which large amounts of amino acids are being rapidly absorbed) is correct, then all the above factors must be taken into account in explaining the figures which we show in Table VII,

of the concentrations of amino acids in the plasma and lymph resulting from feeding with egg-white and fish.

It will be seen in this table that the relative percentages of amino-acids in the cisterna lymph and capillary blood of the villus in fish feeding have the same relations as occur in the peptone experiments;

TABLE VIII. The concentration of the amino acids in the plasma in various vessels and the lymph during peptone and milk (50 c.c. in each experiment) digestion.

			Amino-acid nitrogen (mg. p.c.)				
Cat no.	Grams of peptone	Hours after feeding	Carotid artery	Superior mesenteric vein	Hepatic vein	Inferior vena cava	Lymph
45	20	4	—	20·3	—	—	18·0*
46	20	4	—	19·9	—	—	16·9*
64	20	5	—	15·8	—	—	14·4*
47	15	4	—	19·0	—	—	16·0*
49	15	4	—	24·5	—	—	20·1*
38	10	4	—	18·0	—	—	18·0*
68	10	4	—	12·4	—	—	12·7*
50	5	4	—	15·8	—	—	16·1*
118	5	4	8·0	8·2	6·9	7·6	9·2*
122	5	1	7·7	10·4	7·2	7·7	9·4*

* Cisterna chyli lymph.

whereas when egg-white is fed to the animal these percentages approximate to those seen in the condition of starvation. We are not yet in a position to explain these variations, and until the factors of digestion and emptying of the stomach are worked out, it is perhaps better to refrain from alternative hypotheses. We quote the experiments, however, to show that the question is by no means so simple as appears at first sight, and shall leave these problems for a future communication.

(ii) *Proteins together with fat.* The influence of the admixture of other foodstuffs, for example, fat, may perhaps influence the relationship between amino-acid concentrations in the plasma and chyle or lymph,

TABLE IX. The concentration of the amino acids in the whole blood, plasma and lymph during milk digestion.

		Amino-acid nitrogen (mg. p.c.)				
		Superior mesenteric vein		Hepatic vein		
Cat no.	C.c. of milk	Blood	Plasma	Blood	Plasma	Lymph
34	50	15·3	16·1	14·3	13·3	15·8*
35	50	14·4	15·4	—	—	15·5*
54	150	12·4	11·9	—	—	11·8*

* Cisterna chyli lymph.

as is suggested by Tables VIII and IX, in which peptone and milk, or milk alone, were fed. It will be observed that the percentages here are not dissimilar to those of the egg-white experiment.

Summary and conclusions

1. Observations were made on the concentrations of amino acids in the whole blood and plasma in the carotid artery, superior mesenteric vein, hepatic vein, inferior vena cava and in the lymph from the cisterna chyli or thoracic duct during the conditions of fasting, starvation and feeding with certain foodstuffs.

2. These observations show that the absorption of amino acids from the intestine into the capillaries and lymphatics is in accordance with the physical law of diffusion: there is no clear evidence of selective activity on the part of the capillary or lymphatic endothelium.

3. During the process of rapid absorption of products of peptone digestion the concentration of amino acids in the efferent venous blood of the villus is greater than the concentration in the tissue fluid of the villus, as judged by the efferent lymph, which in turn is greater than the concentration in the afferent arterial blood.

4. The difference between the concentrations of amino acids in the efferent blood and the efferent lymph probably depends on the relative positions of the blood and lymph capillaries, on the velocity of flow in each, and furthermore on the villus tissue fluid being diluted by lymph from the afferent blood capillaries. The blood capillaries lie almost immediately below or even in contact with the covering epithelium, whilst the lacteal is placed centrally and consequently is more remote from the absorbing surface. The rapid flow of blood through the capillaries effects a more frequent change in it than occurs in the lymph.

5. The distribution of the amino-acid concentration in the different representative vessels (carotid artery, superior mesenteric vein, hepatic vein and inferior vena cava) confirms the finding of Van Slyke & Meyer that during active absorption of amino acids by the small intestine, a large proportion of them is broken down during their subsequent passage through the liver.

6. In the fasting or resting state, when absorption of amino acids from the lumen of the intestine is at an end, the relative concentrations of the amino acids in the different vessels mentioned in the preceding paragraph have the same relation to one another, so that the liver is still removing amino acids from the blood passing through it. The relative concentrations of the amino acids in the efferent villous capillaries and tissue spaces alters, the former falling and the latter rising: the relationship thus comes to resemble that in the tissues of the body generally.

7. In the condition of starvation, absorption of amino acids from the lumen of the intestine being absent, the relative concentrations of amino acids in the different vessels referred to in paragraph 5 alter; equalization of concentration between the various vessels occurs except for the inferior vena cava (representing blood from the muscular tissues chiefly) which rises. We interpret this to mean that these tissues are supplying amino acids to the blood in the same way as the intestines do during digestion. The liver still continues to remove amino acids, though at a lessened rate.

8. We are not yet in a position to discuss the absorption of amino acids which result from the digestion of natural foods.

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VITAMIN B_1 AND FATTY LIVERS

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In a previous communication [McHenry, 1935] results have been described showing that choline and vitamin B_1 have a complementary effect in increasing the weights of young rats. In the course of that investigation vitamin B_1 was found markedly to increase the amount of fat in the livers of rats maintained on a low-choline diet. A preliminary report regarding this has been published [McHenry, 1936].

It has been thoroughly established by Best and associates [Best & Huntsman, 1935] that the presence of choline in the diet of rats prevents an increase in liver fat even if the diet contains a great amount of fat. The diets used by these investigators were, in nearly all cases, reasonably adequate with respect to other food constituents. To demonstrate the effect of vitamin B_1 upon the liver fat a diet low in choline must be used. The presence of choline in the diet decreases liver fat and masks the opposite effect shown by vitamin B_1.

Method

In the preliminary report the general type of diet used was described. Purified casein and dried egg-white were employed to furnish protein, the egg-white also providing vitamin B_1. Dietary casein exerts a lipotropic effect similar to that of choline, and dried, alcohol-extracted beef muscle can be used in place of casein. Dried muscle is relatively free from lipotropic effect and, supplied in the same proportion, gives a better rate of growth than does casein. Cane sugar has been consistently used to provide carbohydrate, since it is practically free of choline. For diets containing 30 p.c. or less fat, maize oil is satisfactory as a source of fat. When a greater concentration of fat in the diet is desired maize oil cannot be used since the mixture is too liquid for feeding purposes; in such cases

hydrogenated cotton-seed oil (Crisco) has been employed. The diets have also contained a salt mixture and agar. Vitamins A and D have been furnished as a cod-liver oil concentrate manufactured and assayed by Messrs Ayerst, McKenna and Harrison. The concentrate was dissolved in ether, thoroughly incorporated into the diet mixture, and this was subsequently air dried to remove ether. For those animals receiving choline the required amount of a solution of choline hydrochloride (B.D.H.) was mixed into each day's ration. Two preparations of vitamin B_1 were kindly furnished without charge by Merck, Incorporated, Rahway, New Jersey. The first was the crystalline product prepared by Williams's method, the second a purified concentrate free of choline. Both were assayed by Dr W. Sampson of the Merck Institute for Therapeutic Research. In both cases the required amounts of a solution in 20 p.c. alcohol were mixed with the food. Animals were kept in individual cages with adequate water supply and a daily record of food intake was made. The rats were all albinos of the Wistar strain and were reared in the Connaught Laboratories' colony.

Unless otherwise noted the following basal diet was employed in each experiment:

Casein (B.D.H., vitamin-free) ...	10	p.c. by weight
Egg-white (dried and powdered) ...	5	,,
Cane sugar	48	,,
Maize oil	30	,,
Salt mixture (McCollum's 185) ...	5	,,
Agar	2	,,

For the determination of liver fat the liver was removed under ether anæsthesia or after stunning the animal. The livers were weighed and saponified with potassium hydroxide. The mixture was boiled with alcohol under a reflux condenser, acidified and then extracted with petroleum ether. Aliquot portions of the petroleum ether extract were evaporated to dryness in weighed flasks and the residual fatty acids weighed. All values given are calculated as percentage of total fatty acids in moist tissue. Body fats were determined by the same procedure.

The preliminary experiments were carried out with young female rats and the results, which have been published [McHenry, 1936], showed that the addition of vitamin B_1 to a diet low in choline caused a marked increase in liver fat. This effect has been demonstrated in other experiments in which some 300 rats were employed. Details of some of these will be given.

Results

Observations on young male rats

Twenty animals, weighing initially 50–60 g., were divided into two groups. Individuals in one group received 5γ of crystalline vitamin B_1 per rat per day and those in the second group received no vitamin B_1. Special feeding was continued for 4 weeks. Measurements of body fat were also made.

Group and diet	Average total liver fat p.c.	Average total body fat p.c.
1. Basal diet only	6·2	4·2
2. Basal + vitamin B_1	11·3	6·0

In young male rats the effect of the vitamin is shown as clearly as is the case with young females, and male animals were used in subsequent work since a greater supply was available. The difference in body fat is not great but is interesting, since consistent results have been secured in later experiments.

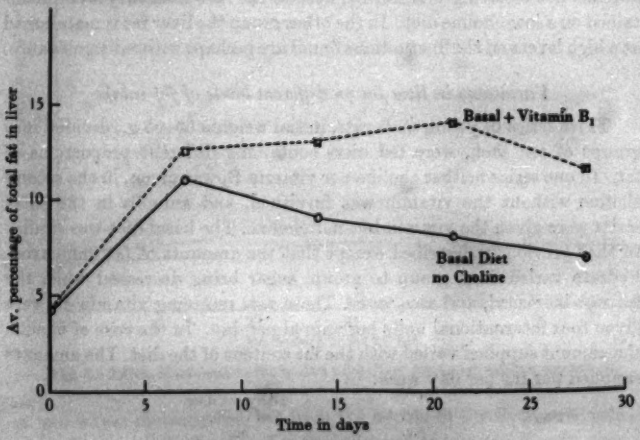

Fig. 1. Rate of production of fatty livers.

Time required for the production of fatty livers

Sixty young male rats, initially weighing from 55 to 65 g., and which had been reared on a complete diet were employed. Ten were killed at the beginning of the experiment and determinations of liver fat were

made. The remaining fifty rats were divided into two groups, one of which received basal diet only while the other was given the basal diet plus four international units of vitamin B_1 per rat per day, supplied from a concentrate. At weekly intervals up to and including 4 weeks equal numbers of rats from each group were killed and liver fats determined. The results are presented in graphical form (Fig. 1). At the end of the first week the amount of liver fat in both groups had approximately doubled and the difference between the groups was not significant. At that time the stores of vitamin B_1 in the animals were presumably not depleted as judged from an examination of the weights. It is interesting that this definite effect of a low-choline diet is shown within 7 days. More recent experiments have shown that the effect of the withdrawal of choline is evidenced in 4 days, and the value of liver fat then secured falls on the line shown in the graph. Apparently supplies of choline in the liver and elsewhere are not available for the prevention of fatty livers. After the first week the amount of liver fat declines in those animals not receiving vitamin B_1, despite the fact that they were maintained on a low-choline diet. In the other group the liver fat is maintained at a high level and the fluctuations found are perhaps without significance.

Variations in liver fat on different levels of fat intake

Three series of young male rats, initial weights 55–65 g., divided into groups of ten each, were fed diets containing different proportions of fat. In one series neither choline nor vitamin B_1 was given, in the second choline without the vitamin was furnished, and animals in the third series were given the vitamin but not choline. The basal diet was similar to that previously described except that the amounts of fat and carbohydrate varied from group to group, sugar being decreased when the fat was increased, and vice versa. Those rats receiving vitamin B_1 were given four international units per animal per day. In the case of choline the amount supplied varied with the fat content of the diet. The amounts supplied per rat per day were:

Dietary fat p.c.	Daily rat dose of choline mg.
0	5
10	5
26	15
42	20
58	30

Fig. 2 shows the average liver fats for each group after 4 weeks' feeding plotted against the percentage of fat in the diet. As would be

expected, choline prevented an increase in liver fat, even when diets high in fat were fed. There is a striking difference between the two series without choline. At all levels of dietary fat the amount of liver fat is markedly increased when vitamin B_1 is fed, and this is true even when there was no fat in the diet. This latter observation may be explained

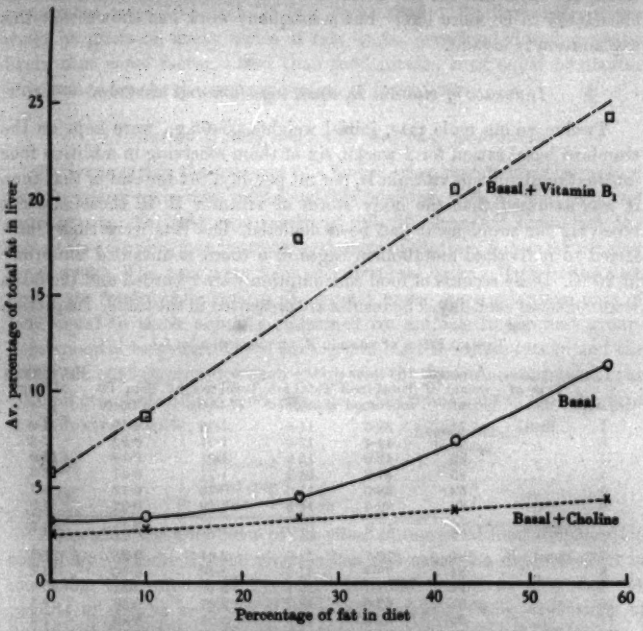

Fig. 2. Effect of amount of fat in diet upon liver fat under various conditions.

in two ways: movement of fat from the depots to the liver, or synthesis of fat from carbohydrate under the influence of vitamin B_1. In view of later experiments the second possibility appears more likely and is in accordance with the results of Whipple & Church [1936]. It is interesting that an appreciable change in the amount of liver fat is not produced by a low-choline diet without a supply of vitamin B_1 until diets containing more than 35 p.c. fat are fed. It seems clear that the ingestion of small

amounts of vitamin B_1 markedly increases liver fat when supplied in a low-choline diet.

In the preliminary report two possible explanations of this effect of vitamin B_1 were mentioned: (1) increased absorption of fat from the intestine, or (2) an effect of the vitamin, directly or indirectly, upon the transport of fat to the liver. At that time the first explanation was considered to be more likely but subsequent work has shown that this explanation is invalid.

Influence of vitamin B_1 upon ingestion and excretion

Twelve young male rats, initial weights 55–65 g., were kept on the standard basal ration for 2 weeks, six of them receiving in addition four international units of vitamin B_1 per rat per day. At the end of that time it was assumed that the body stores of vitamin B_1 in those rats not receiving the supplement had been depleted. The rats were then transferred to individual metabolism cages in a room maintained uniformly at 20° C. Daily records of food consumption were recorded and the faeces were collected each day. The results are presented in the table. Inspection

TABLE. Effect of vitamin B_1 on absorption of fat

Rat no.	Type of diet	Average weight of rat	Total food consumed	Total fat consumed	Total weight of faeces	Total fat in faeces	Fat content of liver p.c.
1	Basal	74	38·5	11·6	1·08	0·19	11·0
2	„	68	44·4	13·3	1·07	0·22	7·2
3	„	95	42·6	12·8	1·01	0·18	15·0
4	„	85	47·8	14·3	1·31	0·27	22·5
5	„	68	38·5	11·6	0·76	0·12	8·7
6	„	69	39·4	14·6	1·19	0·32	7·0
	Average	76	41·9	13·0	1·07	0·22	10·7
7	Basal + B_1	84	76·7	23·0	2·64	0·25	27·9
8	„	81	73·1	21·9	3·16	0·50	13·6
9	„	94	67·1	20·1	2·41	0·41	22·0
10	„	85	62·0	18·7	2·67	0·36	19·3
11	„	85	62·3	18·7	2·09	0·43	7·3
12	„	80	64·2	19·3	2·87	0·61	11·1
	Average	85	67·6	20·3	2·67	0·43	16·9

of this table shows that the food intake of the rats receiving vitamin B_1 was appreciably greater than that of the controls. There is also a difference in the amounts of fat excreted, but the total amount of fat excreted is almost negligible in contrast with the amount eaten, and it appears to be most unlikely that this difference in fat excretion is a factor in the production of fatty livers. However, the increase in food consumption caused by vitamin B_1 might be considered to be of considerable importance.

This is in accordance with the views of Drummond and others that inanition is an important factor in the production of the symptoms frequently associated with a deficiency of vitamin B_1. If one examines the records of the individual animals, however, there is no correlation between fat intake and the amount of fat in the liver. For example, rats 10 and 11 had the same fat intake but the liver fats at the end of the experiment were 19·3 and 7·3 p.c. respectively. Similar observations could be made on every series of rats so far investigated and it seems likely that some factor, other than food intake, is of equal or greater importance for the dietary production of fatty livers.

Production of fatty livers with controlled food intake

To demonstrate the presence of a factor other than food consumption twenty young male rats, initial weights 55–65 g., were divided into two equal groups. The first group received only the standard basal diet, the other this diet plus four international units of vitamin B_1 per rat per day for 4 weeks. The rats were kept in individual cages. Rats in the first group ate freely while rats in the second group were given amounts of food equal to those actually consumed by animals in the first group. Consequently two rats, one of each group, had the same intake and the total food consumption for each group was the same since animals in the vitamin group ate all the food supplied. Determinations of liver fat gave the following results:

Group and diet	Average total liver fat p.c.
1. Basal only	6·8
2. Basal + vitamin B_1	11·1

Even after the avoidance of the effect of increased food consumption caused by vitamin B_1, the vitamin has still exerted a definite effect in increasing the amount of liver fat. It is interesting to note that the records of weight kept for these rats substantiate the conclusions of Sure *et al.* [1932] that vitamin B_1 causes an increase in weight in young rats even with controlled food intake.

In another series of fifty rats, using the same procedure, the following results were secured:

Group and diet	Average total liver fat p.c.	Average total body fat p.c.
1. Basal diet only	7·1	3·6
2. Basal + vitamin B_1	11·6	4·6

Confirmation was thus secured in a large group of animals that vitamin B_1 exerts a specific effect upon fat metabolism not due in any

respect to an increase in food intake. Moreover there was a slight increase in body fat in those animals which showed a marked increase in liver fat.

Effect of vitamin B_1 with a fat-free, high-carbohydrate diet

Forty young male rats were divided into equal groups, one receiving no vitamin B_1, the other four international units per day. The following basal diet was used:

Casein	...	...	10 p.c.	by weight
Egg-white	...	...	5	,,
Salt mixture	...	...	5	,,
Agar	...	...	2	,,
Cane sugar	...	...	78	,,

Cod-liver oil concentrate in ample amounts was also incorporated. The animals were maintained on this regimen for 4 weeks with paired, controlled food intake. The following results were secured:

Group and diet	Average total liver fat p.c.	Average total body fat p.c.
1. Basal diet only	2·8	1·9
2. Basal + vitamin B_1	5·5	2·2

While animals in the second group had approximately twice as much fat in the liver there was also slightly more body fat. It seems likely that the increased amount of fat resulted from synthesis from carbohydrate under the influence of vitamin B_1. Weight records showed that animals receiving the vitamin were appreciably heavier than the controls. Since paired feeding was employed these results cannot be attributed to increase in food consumption. The results confirm the work of Whipple & Church.

Discussion

Recent work by Whipple & Church [1936] has indicated that the principal changes produced by vitamin B_1 in growing rats are increases in the amounts of fat and water in the tissues. In their work the rats were maintained on fat-free diets and it seems likely that the increased fat found in the bodies resulted from synthesis of fat from carbohydrate. Some of the results secured in the present investigation confirm this view. If an increase in fat is the principal change in non-aqueous tissue constituents during growth induced by vitamin B_1, it may well be that an explanation of the complementary effect of the vitamin and choline is provided. It has been clearly demonstrated that choline prevents an

excess accumulation of fat in the liver while vitamin B_1 has an opposite effect. With reference then to fat accumulation in the liver, vitamin B_1 and choline are antagonistic and both may be necessary for normal utilization of fats. One could assume that this is an explanation of their complementary effect in increasing the weights of young rats, since, as has been mentioned, the weight change is due largely to an increase in tissue fat.

The mechanisms of action of choline and of vitamin B_1 in influencing the amount of liver fat have not been elucidated. Part of the response secured when rats are allowed to eat freely appears to be due to an increase in food consumption, but the effect is still secured when the food intake is limited. Results given above show that body fat is increased coincidentally with an increase in liver fat, and this may be regarded as evidence against the supposition that the increased liver fat is due to a depletion of fat from the depots. In a preliminary experiment on hypophysectomized rats the feeding of vitamin B_1 in a low-choline diet failed to cause an increase in liver fat. This is under further investigation.

Summary

1. The oral administration of vitamin B_1 to young male or female rats maintained on a low-choline diet causes a marked increase in liver fat.

2. A similar effect with almost as great intensity is produced in controlled feeding experiments in which the appetite-stimulating effect of vitamin B_1 is avoided.

3. Young rats kept on a low-choline diet without vitamin B_1 exhibit increased liver fat until their stores of vitamin B_1 are presumably exhausted. Subsequently the liver fat diminishes. It can be kept at a high level by the administration of vitamin B_1.

4. The amount of fat in the liver is increased by augmenting the amount of fat in the diet, provided that the diet is low in choline, but the liver fat, at all levels of fat intake investigated, is greatly increased by the ingestion of vitamin B_1. This is also the case when a fat-free, high-carbohydrate diet is employed.

Thanks are expressed to Dr C. H. Best for his helpful interest, and to Miss Lillian Barber and Miss Neta Thompson for technical assistance.

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THE CHEMICAL AGENT IN THE SYMPATHETIC CONTROL OF RETRACTION OF THE NICTITATING MEMBRANE OF THE CAT

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The retractor mechanism of the nictitating membrane (n.m.) of the cat has been shown by Rosenblueth & Bard [1932] to consist of smooth muscle innervated by the cervical sympathetic nerve. Rosenblueth & Cannon [1932] have shown that this mechanism is sensitized by administration of cocaine or by denervation (removal of the superior cervical sympathetic ganglion 4 days previously). The sensitization by ganglionectomy is independent of that produced by cocaine, so that the effects may summate. In a study of the action of drugs on the n.m. Rosenblueth [1932] found that acetylcholine, pilocarpine and physostigmine produced contraction of the membrane, and that the action of these drugs was increased by cocaine and by previous denervation and abolished by atropine. Rosenblueth notes that sympathetic denervation should have no effect on parasympathetic receptors. He also shows that cocaine in these experiments, while sensitizing the action of acetylcholine and pilocarpine on the n.m., did not increase their action on the blood pressure. The similarity in the action of adrenaline and of parasympathomimetic drugs is explained by Rosenblueth on the hypothesis that "the receptor mechanism connected normally with the sympathetic is susceptible of being acted upon by other substances than adrenin including the parasympathomimetic drugs".

Bacq & Fredericq [1935] found that in three out of five cases eserine sensitized to, and atropine reduced the effect of sympathetic nerve stimulation on the n.m. and concluded that in these cases the nerve contained cholinergic fibres.

The evidence cited above suggests that the control of the retractor mechanism of the n.m. in the cat is by some chemical agent resembling that which acts as mediator for the sympathetic supply of the submaxillary gland. It has been shown [Secker, 1934, 1936] that the effects of sympathetic nerve stimulation or of injected adrenaline are enhanced by eserine and inhibited by atropine in sufficient quantity. Feldberg & Guimarãis [1935] in their criticism of the interpretation of the results recorded in the earlier of those papers suggested that the potentiation was due to the action of eserine on the chorda supply of the gland, and not to any peculiarity in the chemical mediator in sympathetic activity whereby it was distinct from adrenaline.

The present investigation was undertaken to study the chemical mediation at the n.m. This organ, according to Rosenblueth, is free from cholinergic nerve supply and, if similar results could be obtained, they would be free from the criticism levelled at the interpretation of the results obtained in the study of the sympathetic control of the submaxillary gland.

Method

In most of the experiments the cats were anæsthetized with sodium barbitone (0·4 g./kg.) intraperitoneally. In a few experiments chloralose following ether induction was used and in others "Dial Liquid Compound, Ciba" (0·08 g. diallyl barbituric acid/kg.). The anæsthetic used did not appear materially to modify the results obtained. Both cervical sympathetic nerves, both vagus nerves and both chorda-lingual nerves were cut and the peripheral end of the sympathetic nerve to be studied was enclosed in shielded electrodes for stimulation by repeated shocks from an inductorium. The contractions of the n.m. were recorded by means of a Starling heart lever, the membrane contracting against the tension of the spiral spring and the direction of pull being controlled by taking a fine silk thread over small pulley wheels. In the later experiments the right membrane was denervated by removal of the superior cervical sympathetic ganglion under ether anæsthesia, at least 7 days being allowed to elapse in order to ensure complete degeneration of the postganglionic fibres. In these experiments the contractions of both membranes were recorded. In some experiments the ducts of both submaxillary glands were cannulated and the secretory responses recorded for comparison with the responses of the membrane. The flow of saliva was recorded by the drop method. Drugs were given by injection into a cannula in the femoral vein. In those experiments in which the suprarenals were excluded from the circulation the method of exclusion adopted

was to tie off both poles of the gland, and then to place a third ligature around the gland and to tie this in such a manner that any neighbouring tissue or vessels became enclosed in a pedicle behind the suprarenal.

Results

Effect of eserine on the response of the n.m. to electrical stimulation of the sympathetic nerve

The stimulus used was always of insufficient strength to cause a maximal contraction of the membrane, and this stimulus was repeated several times to test the uniformity of the response. In most of the experiments the stimulus used was of sufficient intensity to cause marked contraction of the n.m. but was ineffective as a stimulus to salivary secretion. The animal was then given eserine salicylate (0·3 mg./kg.) and after an interval of 15–20 min. the stimulus was repeated. The result of this procedure was always to cause a definite increase in the extent and duration of the contraction of the n.m. and in the secretory response of the submaxillary gland.

Effect of eserine on the response of the n.m. to injected adrenaline

The response of the membrane to injected adrenaline, before and after eserine, was recorded in the same animals with an invariable increase in the extent and duration of the response after eserine. Figs. 1 and 2 are typical records of the effect of eserine on the responses of the n.m. to nerve stimulation and to adrenaline respectively. The small variations superimposed on the main record in Fig. 1B are due to spontaneous eye movements which sometimes occur after eserine. That these movements are not concerned in the potentiating effect is shown in later figures recording potentiation in their absence.

Effect of eserine on the n.m. previously sensitized with cocaine

Administration of cocaine hydrochloride in doses up to 8 mg./kg. gave a definite potentiation of the response of the n.m. to nerve stimulation or to injected adrenaline; this potentiation was further increased by the action of eserine (Figs. 3 and 4). This double sensitization of the mechanism by cocaine and by eserine would seem to suggest a dual nerve supply to the membrane, the adrenergic supply having its action increased by cocaine and the cholinergic supply being affected in a similar way by the eserine.

Fig. 1. Contraction of the n.m. on stimulation of the cervical sympathetic nerve. (A) before, and (B) after eserine.

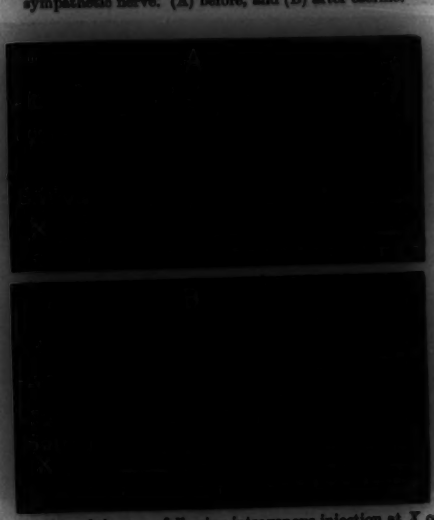

Fig. 2. Contraction of the n.m. following intravenous injection at *X* of adrenaline (0·05 mg./kg.). (A) before, and (B) after eserine.

Fig. 3. Contraction of the n.m. on stimulation of the sympathetic nerve at X. (A) normal, (B) after cocaine (5 mg./kg.), (C) after cocaine and eserine.

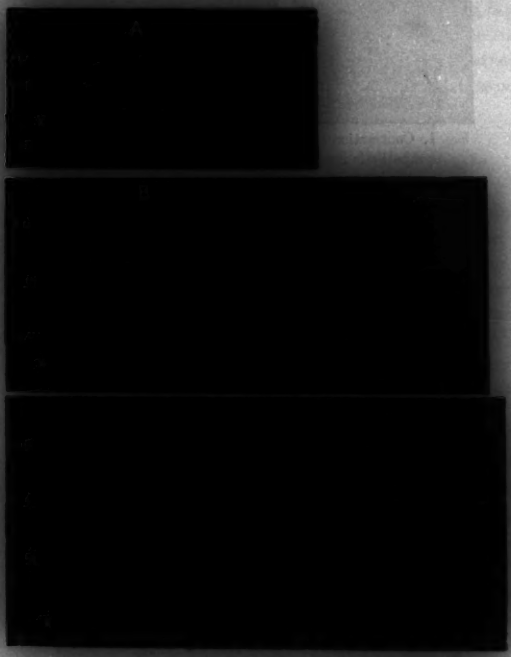

Fig. 4. Contraction of the n.m. following adrenaline (0·05 mg./kg.) given at X. (A) normal, (B) after cocaine (5 mg./kg.), (C) after cocaine and eserine.

Mode of action of eserine

It is to be noted that in none of these experiments did the injection of eserine salicylate (0·3 mg./kg.) produce of itself any response by a normally innervated membrane. In two experiments out of six a small contraction was produced in the denervated membrane, but this passed off before the end of the usual interval of 20 min.

A possible explanation of the potentiation of sympathetic activities by eserine is that the effect may be due not to any action of the drug on the n.m. but to its known action at the suprarenals, causing an output

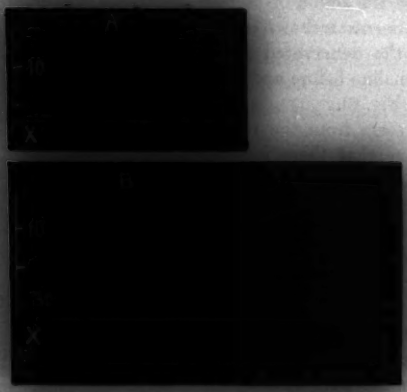

Fig. 5. Contraction of n.m. following adrenaline (0·05 mg./kg.) given at *X* in adrenalectomized cat. (A) before, and (B) after eserine.

of adrenaline in quantities too small to cause contraction of the membrane but sufficient to lower the threshold to subsequent stimuli. In order to test this hypothesis the previous experiments were repeated on cats after exclusion of the suprarenals. These tests showed clearly that adrenalectomy was without effect on the potentiating action of eserine on the response to adrenaline (Fig. 5).

When, however, the response of the n.m. to nerve stimulation was studied after adrenalectomy it was found that in almost every experiment the sensitivity of the retractor mechanism gradually declined, so that in many cases no contraction resulted from nerve stimulation no matter

what the intensity of the stimulus. Since this fading occurred before eserine was given and progressed after that drug had been administered, it was difficult to demonstrate its potentiating action on the effects of nerve stimulation. In two experiments, however, it was possible to show that adrenalectomy had not interfered with this action. The question of the progressive failure of the mechanism to nerve stimulation after adrenalectomy is being investigated and will be reported later.

There still remains the possibility that the potentiation might be due to the protection which eserine affords to acetylcholine, allowing this substance greater play at the synapses of the superior cervical ganglion. That this latter is not the explanation of the action of eserine in these experiments is shown by the responses of the denervated membrane to adrenaline before and after eserinization (Fig. 6).

Fig. 6. Contraction of denervated n.m. to adrenaline (5 μg./kg.) given at *X*. (A) before, and (B) after eserine.

It would seem from the results recorded above that there is some mechanism in the n.m. itself in which the exciting agent, adrenaline on the one hand, or the product of the impulses of the sympathetic nerve on the other, has its effectiveness enhanced by eserine. The potentiation of the effects of nerve stimulation by eserine suggest that the nerve contains cholinergic fibres, but such an interpretation does not explain the fact that the response of the membrane to adrenaline is also enhanced. If, as stated by **Rosenblueth & Bard**, the retractor mechanism receives its entire nerve supply from the cervical sympathetic nerve, and we accept also the view of **Bacq & Fredericq** that this nerve supply contains some cholinergic fibres, then the smooth muscle must have a dual nerve supply and be capable of giving a common response to stimulation of either adrenergic or cholinergic fibres. If these conditions exist it might be possible that, under the influence of eserine, subthreshold quantities of acetylcholine are becoming available at the terminals of the cholinergic fibres and that, while being insufficient in quantity to cause contraction of the membrane, they are responsible for the potentiation

of the action of adrenaline. The difficulties in the way of accepting this interpretation are:

(1) The fact that the cervical sympathetic nerve is cut and that therefore no impulses are passing which could cause any liberation of acetylcholine.

(2) The persistence of the phenomenon in the membrane denervated 8 days previously by ganglionectomy. On this interpretation it would be necessary to assume that eserine itself was capable of causing the liberation and accumulation of minute traces of acetylcholine in the n.m.

(3) The results which follow intravenous or intra-arterial injection of acetylcholine do not lend any support to the hypothesis.

Effect of acetylcholine injections on the n.m.

Intravenous injection of acetylcholine bromide in doses varying from 1–5 μg./kg., while producing marked effect on the vascular mechanism, were without effect on the n.m. even after the animal had been given eserine. In a small number of experiments the acetylcholine was injected into a cannula in the lingual artery to give immediate entry into the carotid artery; here again the drug was ineffective unless given in doses of 15–20 μg./kg. when a small contraction of the membrane sometimes occurred. In a few experiments the action of acetylcholine on the membrane sensitized by previous denervation was tested, again without response unless large doses of the drug were given. When the sensitivity was still further increased by the action of eserine a slight contraction of the membrane followed the giving of fairly large doses of acetylcholine. The response in these cases was always very slight. Fig. 7 is a record of the effect of acetylcholine 20 μg./kg. given intravenously. The tracing A shows the failure of response on the part of both the normally innervated left membrane and the denervated right membrane. Tracing B shows the effect of eserine on the response and in this case the sensitive right membrane has given a small response, but the normal left membrane was unaffected. The response given by the denervated membrane in this case is of the order of what would be obtained from the intravenous injection of 0·5 μg./kg. adrenaline. The lack of sensitivity of the n.m. to acetylcholine is further shown by the lack of any response on the part of the membrane, sensitized by previous denervation and by eserine, to acetylcholine liberated into the blood stream when a cholinergic nerve such as the chorda-lingual is stimulated, and the acetylcholine escaping is of sufficient quantity to cause a marked fall in the blood pressure.

Finally, it may be stated that where acetylcholine has been injected into the blood stream of an eserinized animal with marked vascular response, but without effect on the n.m., the contraction following an immediate injection of adrenaline shows no increase over that resulting from a similar dose of adrenaline not preceded by acetylcholine.

It seems justifiable to conclude from these results that while the n.m. is capable of responding to large doses of acetylcholine injected into the blood stream it is insensitive to quantities which may be justly considered to be greater than physiological amounts, and that therefore this substance is not concerned in the potentiation by eserine of the response of the n.m.

Fig. 7.

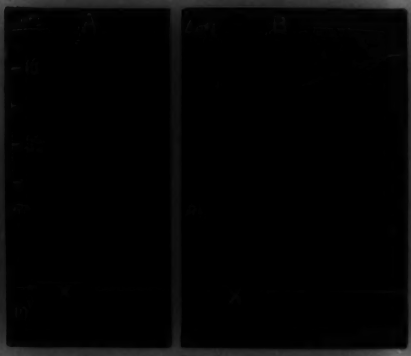

Fig. 8.

Fig. 7. The effect of intravenous acetylcholine (20 μg./kg.) at *X*. (A) before, and (B) after eserine. For description see text.

Fig. 8. The effect on the denervated right membrane of stimulation at *X* of the left sympathetic nerve. (A) before, and (B) after eserine.

Eserine and the distant effects of the chemical mediator of sympathetic activity

In a series of experiments, using cats in which the right n.m. had been sensitized by ganglionectomy, the effect on that membrane of stimulation of the left cervical sympathetic nerve was studied. In most of the

Fig. 9. The responses of the n.m. and submaxillary gland to adrenaline (0·05 mg./kg.) given at *X*. (A) before, and (B) after atropine sulphate (1 mg./kg.).

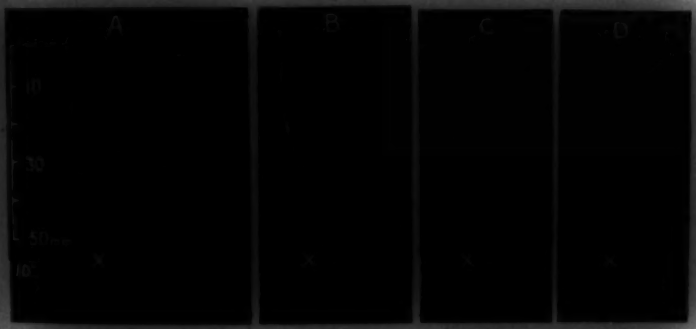

Fig. 10. The influence of atropine on the contractions of the n.m. following stimulation of the sympathetic nerve at *X*. (A) normal, (B) after 0·3 mg./kg., (C) after 0·6 mg./kg., (D) after 4 mg./kg. atropine.

experiments this procedure was without effect, but in a few the denervated membrane gave a very slight contraction. The cats were now given eserine salicylate (0·3 mg./kg.) and when after the usual interval of 20 min. the same strength of stimulus was applied to the left nerve, the right membrane, if previously inactive, now contracted, or, if previously active had its response increased. Fig. 8 is a record of one such experiment in which the denervated membrane failed to respond before eserine was given. The record also shows the potentiation of the response of the normally innervated membrane by the eserine. No such crossed response was obtained in any experiment in which the detector membrane had not been sensitized by ganglionectomy.

The effect of atropine on the responses of the n.m.

The response of the n.m. to stimulation of the sympathetic nerve or to injected adrenaline is markedly diminished by atropine sulphate in doses of 0·5 mg./kg. There is a progressive failure of the responses with increasing dosage of atropine, but very large doses (20 mg./kg.) are required to give complete abolition of the responses. The effect of atropine sulphate (1 mg./kg.) on the response to adrenaline (0·05 mg./kg.) is shown in Fig. 9, and the effect of increasing doses on the response to nerve stimulation is recorded in Fig. 10.

Discussion

The results recorded above show that the retractor mechanism of the n.m. of the cat, excited by stimulation of the cervical sympathetic nerve or by injected adrenaline, has its responsiveness increased by eserine and diminished by atropine. These facts in their application to the effects of nerve stimulation would be accounted for if it could be shown that the nerve contained a small number of cholinergic fibres. Such demonstration would not, however, account for the effects recorded in the responses to injected adrenaline. The interference of acetylcholine at any point in the transmission of excitation to the smooth muscle of the membrane would appear to be unlikely in the light of the results obtained when this substance is injected into the blood stream.

The possibility that the potentiation of the nerve effect might be due to the action of eserine in protecting acetylcholine at the synapses in the superior cervical sympathetic ganglion has not been directly excluded. In view of the results obtained with the denervated membrane, however, we can discount this possibility, unless we postulate a mechanism

for the action of adrenaline entirely different from that for the effects of nerve stimulation. Finally the diminution of the responses after atropine is not of the order which would be expected if acetylcholine were the transmitting agent. If we were dealing with conditions in which a few cholinergic fibres were present in the nerve supply, then those fibres would have their action inhibited by the first small dose of atropine and further dosage with the drug should not further diminish the responses.

These experimental facts would seem to justify the conclusion that the chemical mediator of sympathetic activity at the n.m. is not acetylcholine, but some other substance the action of which is enhanced by eserine and retarded by atropine. The site of action of the eserine would seem to be peripheral to the terminals of the postganglionic fibres, since the effects of the transmitter liberated by the left sympathetic nerve and carried in the blood stream to the right denervated membrane are increased by the presence of the drug.

The mode of action of eserine and of atropine in these experiments is not obvious. The action of eserine is certainly to increase the magnitude of the contraction of the membrane and to prolong that contraction, but there is nothing in the results obtained to indicate whether the action is one of protection of the chemical mediator, as in the case of protection of acetylcholine by inhibition of the esterase, or whether it is some direct action of the eserine on the smooth muscle of the membrane whereby its general excitability is increased, or it is made more susceptible to some specific chemical agent liberated on sympathetic excitation. Until this latter question is answered there is no point in speculating on the nature of the chemical agent in the activity. It is clear however that the retractor mechanism of the n.m. resembles the sympathetic excitor mechanism for secretion in the submaxillary gland. This suggests that the results obtained in the experiments on salivary secretion [Secker, 1934, 1936] were independent of the fact that the gland was in receipt of a dual nerve supply.

Summary

1. The responses of the nictitating membrane of the cat to nerve stimulation or to adrenaline are potentiated by eserine and diminished by atropine.

2. The potentiation is still apparent after adrenalectomy and after denervation of the membrane by ganglionectomy 8 days previously.

3. The distant effects of the chemical mediator of sympathetic activity are increased by eserine.

4. The evidence given seems to justify the conclusion that while acetylcholine in large quantities may cause some contraction of the membrane, this substance is not responsible for the results obtained in the experiments.

5. The observations show that the excitor mechanism in the n.m. of the cat resembles the sympathetic mechanism in the submaxillary gland.

I wish to record my thanks to Prof. Burns for his interest and criticism during the course of this work. I have also to acknowledge, with thanks, that the expenses involved were, in part, defrayed by a grant from the Medical Research Council.

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612.627:612.492.8

THE INSENSITIVITY OF THE CERVIX UTERI TO OXYTOCIN

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In spite of the fact that parturition can occur in the absence of the pituitary gland, and although the oxytocic principle of its posterior lobe must still rank as an extract rather than as a hormone, it would be rash to state that this principle had nothing to do with parturition. Robson has advanced evidence that in the rabbit [1933*a*], human [1933*b*] and mouse [1934], the threshold of uterine muscle to the oxytocic principle becomes progressively lower as pregnancy advances, while the clinical use of the extract, so suggestive of specific function, is too well known to need description. Nevertheless, definite evidence proving that the principle is concerned with parturition is lacking.

If such evidence is ever brought forward, it must take account of the fact that mere contraction of the uterus is too primitive a process to be dignified by the name of parturition: it is impossible, for instance, to visualize the delivery of ten fœtuses, five in each horn of the uterus, without some co-ordinating mechanism. It is not necessary to endow the oxytocic principle itself with any co-ordinating function, for the type of muscular activity might be determined by some other factor, the oxytocic principle serving merely to potentiate the contractions. It is, on the other hand, necessary to postulate that this should not cause inco-ordinated movements.

The most severe, and at the same time the most easily demonstrable, type of inco-ordination would be a simultaneous contraction of the cervix and cornua of the uterus, and in the author's opinion this would, if brought about by the oxytocic principle, definitely settle all doubts on the score of its physiological activity. Experiments on the uterus of the pregnant goat [Newton, 1934] offered no such easy way out of the

difficulty. The corpus and cervix uteri exhibited not only opposite reactions to adrenaline, suggesting reciprocal innervation, but the cervix proved quite insensitive to "Pitocin" (Parke, Davis and Co.). A number of sheep uteri were then obtained with the object of extending the experiments, but the "cervix" was so constructed anatomically as to make them useless for the purpose. It was therefore determined to return to smaller animals and to use a method of highly magnifying the contractions of the cervix to make them visible. In this paper the results of experiments on guinea-pigs and rats are recorded.

Method

The animals were killed by a blow on the head and bled. The abdomen was opened, the vagina cut transversely and the cervix of the uterus cut off with a pair of sharp scissors, giving a ring of tissue. In most of the guinea-pig experiments as much as possible of the outer covering of mucous membrane was cleaned off the muscle so that this was freely exposed to the actions of substances in solution. The cervical ring was suspended in physiological saline solution [Burn & Dale, 1922] in the usual way by two hooks, the lower fixed to the oxygen supply tube, the upper movable and hanging by a thread from a small lever (about 1 cm. in length), whose movement rotated a small mirror at the fulcrum. The movements of the mirror were recorded optically on photographic paper, the amplitude of the uterine contractions being magnified 175 times.

"Pitocin" (Parke, Davis and Co.) was used as the oxytocic principle, and was diluted with the same solution as that in the muscle bath, so that the usual volume added was 0·1–0·5 c.c.

Results

In these experiments eighteen guinea-pigs were used, two being pregnant, two injected with œstrin, and six in natural œstrus. The state of the vagina had been recorded daily for several months in the non-pregnant animals, and the first day of opening was regarded as a sufficiently close approximation to œstrus for these experiments. Vaginal smears were not used, and it is quite possible that the animals were not all killed at the "peak" of œstrus which is short in the guinea-pig.

In only one animal did Pitocin seem to have a slight stimulating action on the cervix (Fig. 2*a*), in spite of the fact that after the first three experiments the cervix was tested, usually without preliminary trial, with a dose of 2 international units added to a bath of 50 c.c. saline.

The power of the cervix to contract was demonstrated by the effect of 1 in one million or 1 in half a million adrenaline hydrochloride solution (Fig. 1). In a few experiments a tendency to diminished activity was apparent after the large dose of Pitocin, and this was noticed particularly in two guinea-pigs in œstrus; the tracing from one of them is shown in

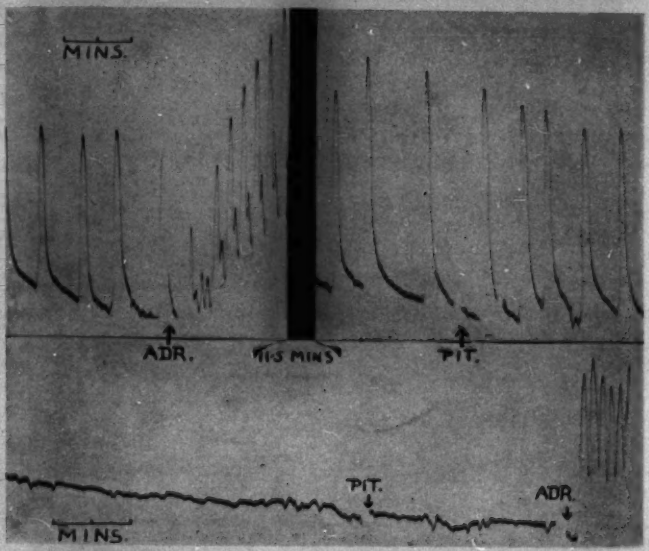

Fig. 1. Contractions of cervix of non-pregnant guinea-pig. The upper part of the tracing shows the actions of 1 in half a million adrenaline (*ADR*) and 2 units of Pitocin in 50 c.c. (*PIT*). The lower tracing shows the disappearance of spontaneous activity due to changing the solution twice. *PIT* and *ADR* have the same meaning as before. The lower tracing is typical of the normal behaviour of many cervical strips.

Fig. 2*b*. To ascertain whether this had anything to do with œstrus (though not apparent in other œstrous animals), two guinea-pigs were treated beforehand with very large doses of œstrin, but they each gave an inactive type of tracing (Fig. 3) from the cervix, and this was now inert to adrenaline as well as to Pitocin. (The dose of œstrin was approximately 50 times that which would be necessary to bring a guinea-pig into full œstrus, but, although it is realized that this may have had

secondary effects, the indications that "depression" after Pitocin was associated with œstrus were deemed too slight to justify experiments on oophorectomized hypophysectomized animals.) The Pitocin used was made up to *p*H 3·9 with acetate buffer solution, and was not guaranteed

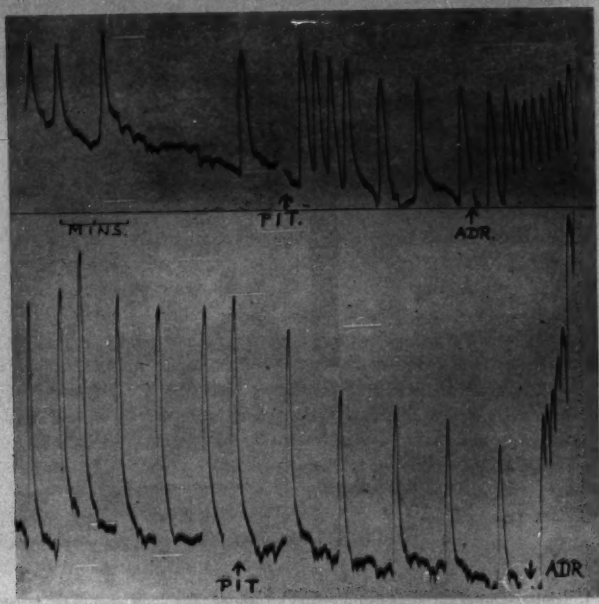

Fig. 2. *PIT* = 2 units Pitocin in 50 c.c. *ADR* = 1 in a million adrenaline.

(*a*) Upper tracing. Cervix of non-pregnant guinea-pig showing apparent transient stimulation by Pitocin. Solution not changed between *PIT* and *ADR*.

(*b*) Lower tracing. Cervix of non-pregnant guinea-pig, in œstrus, showing apparent depressing effect of Pitocin. Solution not changed between *PIT* and *ADR*.

by the manufacturers to be absolutely free from traces of protein; the large dose of 2 units required 0·2 c.c. of this solution, and as the muscle bath had a capacity of only 50 c.c., it is highly probable that slight changes in the behaviour of the cervix, in either direction, were non-specific in origin. It may also be assumed, with a fair degree of certainty, that a slight, occasional, and variable change which requires such a high

concentration of Pitocin cannot be of physiological importance. In all animals in which it was tested (10 out of 18), the longitudinal muscle taken in strips of about 2 mm. length from the horns was sensitive to less than 0·1 unit of Pitocin, usually to 0·02 or 0·04 unit (Fig. 3). The circular muscle also responded to Pitocin in doses of the same order.

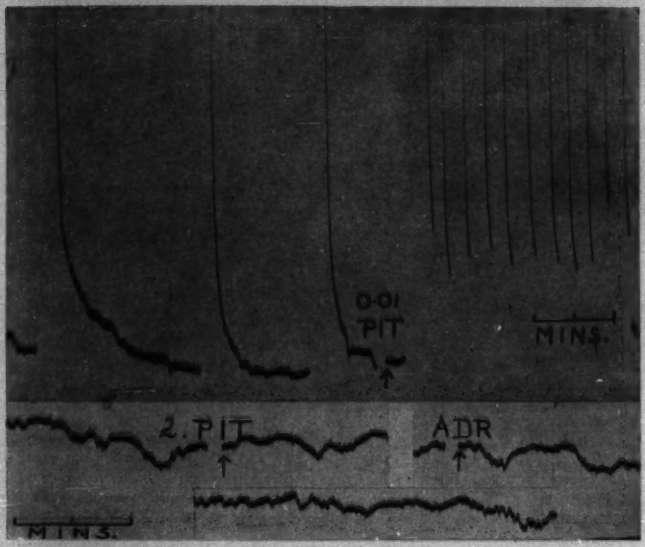

Fig. 3. Non-pregnant guinea-pig injected for 3 days previously with a total of 5 mg. dihydroxy œstrin benzoate (Schering). Upper tracing: longitudinal strip from horn, showing effect of 0·01 unit Pitocin (0·01 *PIT*). Middle tracing: cervix, showing insensitivity to both 2 units Pitocin (2 *PIT*) and 1 in half a million adrenaline (*ADR*). Bottom tracing: that part of the middle tracing omitted between 2 *PIT* and *ADR*.

The stage of gestation was unknown in the two pregnant animals, but in the first, implantation had clearly just occurred, while in the second, the fœtuses were half the length they attain at birth. In neither case did the cervix contract on treatment with 2 units of oxytocin; that from the guinea-pig more advanced in pregnancy had lost its reactivity to adrenaline.

Identical experiments were made with six rats, three non-pregnant (one in œstrus) and three pregnant. Two units of Pitocin had no effect on the cervix of any of these, and although the ability to contract could not be exhibited by giving adrenaline, the spontaneous variations in tonus were sufficient to show that the muscle could have contracted had it been stimulated (Fig. 4). The uterine horns of such animals are

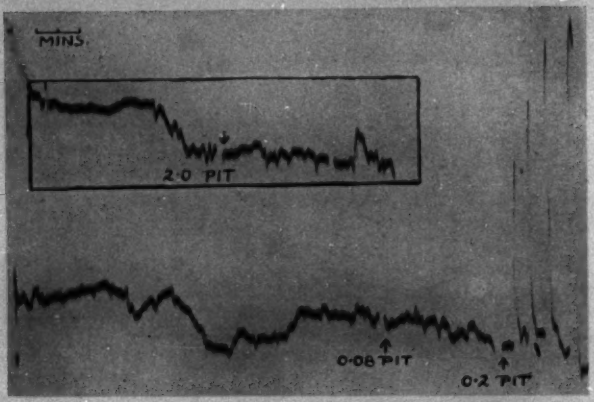

Fig. 4. Rat, 13th day of pregnancy. Main tracing: longitudinal strip from horn, showing effect of 0·08 and 0·2 unit of Pitocin (0·08 *PIT* and 0·2 *PIT*). Solution not changed between the doses. Inset tracing: ring of cervix, showing insensitivity to 2 units Pitocin (2·0 *PIT*), although a spontaneous relaxation had just occurred, indicating that the muscle was not already fully contracted at the time of administering Pitocin.

sensitive to 0·02–0·2 unit of Pitocin, but strips small enough for optical recording are sometimes difficult or impossible to stimulate. It seems that the muscle of the horns is not uniformly responsive to Pitocin.

Conclusions

The implications of these experiments were discussed at the beginning of the paper and the conclusion from them, and from those on the goat previously reported, is that the oxytocic principle of the pituitary gland still cannot be excluded from the factors responsible for parturition. If anything, the possibility that it plays a part is enhanced.

Summary

Experiments are described showing that in the rat and guinea-pig, in any phase of sexual activity, the cervix uteri does not react *in vitro* to large doses of Pitocin.

I wish to thank Mr J. B. Brooksby for his assistance in the last stages of this work. The expenses were defrayed by a grant from the Medical Research Council.

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Robson, J. M. (1934). *Ibid.* **82**, 105.

Note added in proof. Following a query by one of the Editors, further experiments were performed with œstrous guinea-pigs, œstrus being carefully checked by the vaginal smear. As before, 2 units of Pitocin were without effect on the cervical muscle, but adrenaline, in the same doses as previously, increased the amplitude of the spontaneous contractions without affecting the tonus of the muscle. This is probably a stage in the complete abolition of the adrenaline effect which follows excessive dosage with œstrin. Although the actions of adrenaline are merely incidental to this investigation, it may appear that the further absence of effect noticed during pregnancy is evidence of the presence of considerable quantities of œstrin in the body at this time. The behaviour of the uterine horns does not confirm this impression, for they are relaxed by adrenaline during dioestrus and pregnancy (Gunn, J. A. and Gunn, J. W. C. (1914) *J. Pharmacol, Baltimore*, **5**, 527) and contracted by it during œstrus and after œstrin injection (Kochman, M. and Seel, H. (1929) *Z. exper. Med.* **68**, 238).

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THE ACTION OF CERTAIN ENZYME POISONS ON THE FROG'S AURICLE

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The original object of the experiments described in this paper was to determine whether it was possible to observe any difference between the actions of pure asphyxia and of cyanide on the frog's heart poisoned with iodo-acetic acid (I.A.A.). It was hoped in this way to decide whether, in this tissue, the oxygen uptake is entirely abolished by cyanide, as has been found by Warburg and his school for various cell suspensions and some mammalian tissues [Warburg, 1923, 1931; Alt, 1930], or whether, as found by Dixon and his collaborators [Dixon & Thurlow, 1925; Dixon & Elliott, 1929], working with mammalian tissues, there is always a residual oxygen uptake whatever the concentration of cyanide used. It seemed possible that if there were a residual oxygen uptake, it might be sufficiently large to maintain small contractions. Though this was not found to be so, indirect evidence was obtained in support of Dixon's findings.

The I.A.A.-poisoned heart was chosen for this study because this preparation when asphyxiated fails through rapid depletion of its ultimate store of energy, whereas in the normal heart asphyxia only causes failure if the lactic acid produced under these conditions is allowed to accumulate and so inhibit further carbohydrate breakdown [Clark *et al.* 1932, 1934].

Cyanide being an oxidase poison, experiments were carried out to determine whether it causes failure in virtue of this property. The action of a dehydrogenase poison (ethyl urethane) was also investigated.

Experimental methods

The tissue chosen for these experiments was a strip of frog's auricle. This preparation when carefully freed from all sinus tissue seldom beats spontaneously and can therefore be stimulated artificially at any desired

rhythm. It has the further great advantage that, being thin, it receives sufficient oxygen when simply suspended in a bath of aerated Ringer's solution. The bath in these experiments was a glass tube 5 cm. in length and 1 cm. in diameter closed at its lower end by a rubber bung. The bung was pierced by a hypodermic needle through which air was blown to oxygenate the Ringer's solution, and also by a stout silver wire. The latter formed one of the stimulating electrodes, and its upper end was bent into a loop to which the lower end of the strip was attached with a small loop of thread. The second electrode was another piece of the same silver wire which dipped into the Ringer's solution. Rhythmic stimuli were provided by an induction coil connected to a rotary contact breaker, and the normal rate of stimulation was 15 per min. The upper end of the strip was connected by a fine thread to a light straw lever writing on a smoked drum. It was found necessary to smoke the drum paper very lightly, as a thick layer of soot on the drum impeded the movement of the writing point and the record then obtained was that of an incomplete excursion of the lever. The Ringer's solution used had the following percentage composition: NaCl 0·65, KCl 0·015, $CaCl_2$ 0·02, Na_2HPO_4 0·05, and its *p*H was adjusted to 7·5. It was found in the first few experiments that the auricular strip rapidly fails when it is poisoned with I.A.A., and this was shown to be the result of a depletion of the strip's stores of energy-producing substance, for it recovered immediately if a little sodium lactate was added to the Ringer's solution bathing it. In all subsequent experiments in which the strip was poisoned with I.A.A., 0·1 p.c. sodium lactate was added to the Ringer's solution when it was made up at the beginning of the experiment, and the strip then beat vigorously for many hours. The concentration of I.A.A. used for poisoning the strips was 1 in 10,000, and this was allowed to act for 30 min. after which it was replaced with normal Ringer's solution.

The sodium cyanide solutions were freshly made up each day from the crystalline salt to avoid the risk of a change of concentration by decomposition. The salt was dissolved in distilled water, and the resulting solution which was strongly alkaline was brought to *p*H 7·5 by addition of hydrochloric acid, and then placed in a tube which was tightly stoppered to prevent the escape of the HCN liberated by the addition of the acid. In those experiments in which the action of cyanide was to be studied quantitatively it was necessary to add the cyanide solution to the Ringer's solution in such a way that the addition could be made instantaneously at a moment which could be marked on the smoked drum. The solution was therefore made up in a concentration a hundred times that whose action

was to be studied, so that to obtain the desired ultimate concentration a volume one-hundredth of that of the fluid bathing the strip was added to the bath. The volume of the fluid in the bath being 3 c.c., 0·03 c.c. of the cyanide solution was added from a micro-pipette. In this way the fluid was added instantaneously and without disturbing the strip, and the stream of air bubbling through the Ringer's solution produced rapid and thorough mixing. Precautions had to be taken to avoid a diminution in the concentration of cyanide in the bath resulting from a removal of free HCN by the stream of air. The air was therefore passed on its way to the bath through a solution of cyanide of the same concentration as that under investigation, so that, when it reached the bath, it contained the same tension of HCN as the Ringer's solution. It was also necessary to ensure that the cyanide was thoroughly washed out of the strip after one observation before making another. The washing was accomplished by changing the Ringer's solution several times, varying from three times after the low concentrations of cyanide, to seven or eight times after the higher concentrations. With the highest concentrations of cyanide it was further found necessary to stop the rhythmic stimulation when washing was begun, as it was not possible to wash out the cyanide quickly enough to stop the strip going into rigor unless this was done. Further, since Clark [1935*a*] found that cardiac muscle poisoned with I.A.A. requires at least 20 min. to recover from asphyxia, in that a second asphyxia carried out before this time has elapsed causes a much more rapid fall of the mechanical response, half an hour was always allowed to elapse between two applications of cyanide in the present experiments.

Results

The action of cyanide on the I.A.A.-poisoned auricle

It was thought advisable before beginning the experiments on the I.A.A.-poisoned auricle to do a preliminary control experiment on the normal strip to ascertain whether cyanide had any toxic action which might be confused with its asphyxiating action. If its action was simply an abolition of oxidative processes its only action on the normal strip immersed in alkaline Ringer's solution should be a slight initial fall of the mechanical response leading to a prolonged gradual fall resulting from depletion of the stores of carbohydrate in the muscle, and this was found to be so (see Fig. 4*a*). It was therefore concluded that the cyanide had no toxic action of any significance.

Since cyanides inhibit the uptake of oxygen by tissues it was to be expected that their action on the I.A.A.-poisoned heart would be similar

to that of simple asphyxia produced by driving all the oxygen out of the perfusion fluid by a stream of nitrogen. Fig. 1 shows that this is the case, the only difference between the two curves being that the cyanide acts more rapidly than the nitrogen, no doubt because the penetration of the tissue by cyanide is a quicker process than the displacement of oxygen by nitrogen. In this experiment the concentration of cyanide used, *M*/500, was sufficient to produce abolition of contraction leading to contracture.[1] A few experiments were made with a view to determining the minimum concentration which could produce this effect, and

Fig. 1. I.A.A.-poisoned frog's auricular strip: *a*, at arrow *M*/500 NaCN in bath; *b*, at arrow N_2 on. Time in min.

observing the action of concentrations below this minimum. Fig. 2 shows a series of curves obtained in such an experiment, and it is evident that a concentration of *M*/2500 produces the maximum effect, while concentrations below *M*/2500, in this experiment *M*/5000, *M*/10,000 and *M*/15,000, produce partial inhibition of contraction, namely 95, 60 and 40 p.c. respectively. It might appear at first sight reasonable to conclude from these results that concentrations of cyanide, which have the same effect as complete asphyxia produced by removal of oxygen from the tissue, must cause a complete inhibition of oxygen uptake by the tissue. Such a conclusion would be in agreement with the findings of Warburg and his collaborators, but in disagreement with the results obtained by Dixon & Elliott [1929], and by Kisch [1933]. Further consideration, however, shows that we cannot legitimately draw such a conclusion, for

[1] In the tracings shown in the figure the cyanide and nitrogen were not allowed to act long enough to produce complete abolition of contraction, as recovery of the muscle after such treatment is prolonged and incomplete and the making of both observations on the same strip in good condition is thus impossible.

to abolish the contractions of a muscle we need only reduce the oxygen uptake to that required by the resting muscle. Now it has been shown by many investigators [Weizsäcker, 1912; Clark & White, 1928, 1929; Clark, 1935*a*] that the resting metabolism of frog's cardiac muscle is high relative to that of the contracting muscle, the most recent estimate given by Clark [1935*a*] being that the metabolism of the resting

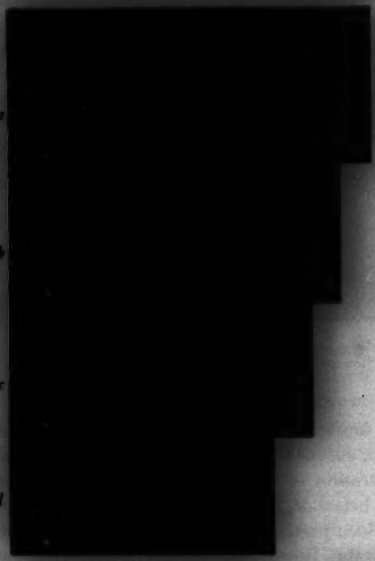

Fig. 2. Effect on I.A.A.-poisoned frog's auricular strip of various concentrations of NaCN: *a*, *M*/15,000; *b*, *M*/10,000; *c*, *M*/5000; *d*, *M*/2500. Time in min.

frog's ventricle is 25 p.c. of that of the ventricle beating 15 times per min. Since in the present experiments the rate of stimulation was 15 per min., and assuming that the relation between resting and active metabolism is the same for the auricle as for the ventricle, all we are entitled to conclude is that the concentrations of cyanide which abolish the contractions reduce the oxygen consumption by at least 75 p.c. The results of the next series of experiments show, moreover, that the oxygen consumption of the frog's auricle is not completely abolished by cyanide, even in a concentration as high as *M*/150.

These experiments were carried out according to a method devised by Clark [1935*a*] for determining indirectly the resting metabolic rate of frog's ventricular muscle. The method consisted in determining, at various rates of beating, the rate of fall of the mechanical response produced by asphyxiating the I.A.A.-poisoned ventricle. The reciprocal of the time required to produce a fall of the mechanical response to half its normal value was taken as a measure of the rate of fall, and since in a muscle under these conditions the fall in the mechanical response is the result of the progressive depletion of a store of energy-producing material, this reciprocal was also a measure of the rate of depletion of this store, i.e. of the metabolic rate of the muscle. These reciprocal values when plotted against the corresponding rates of beating gave a series of points lying on a straight line, which when produced cut the ordinate at a point which gave a value for the metabolic rate corresponding to zero rate of beating, i.e. the resting metabolic rate. As mentioned above, Clark found this rate to be about 25 p.c. of that of the ventricle beating at 15 per min. Now it is obvious that this method is only valid for determining the resting metabolism if the method of producing asphyxia completely abolishes the uptake of oxygen by the muscle, so that the muscle is relying for its energy supply on a store which is not being replenished. If the asphyxia is incomplete, as will occur if the nitrogen used is contaminated with a small percentage of oxygen, some of the energy-producing substance broken down as a result of contraction can be reformed, the store of the substance will not be depleted as rapidly as it is when asphyxia is complete, and the rate of fall of the mechanical response will be correspondingly less. Moreover this effect will be most apparent at low rates of beating, for with a long interval between beats there is a greater likelihood of completion of the partial recovery than with a short interval. If the results from such an experiment are plotted we should therefore expect that the slope of the line joining the points will be steeper than that of one obtained from an experiment in which asphyxia has been complete, and it will cut the ordinate at a point corresponding to a value less than that of the resting metabolic rate. This argument may now be used conversely, for if, on plotting the results of an experiment, we find that the line joining the points cuts the ordinate at a point corresponding to a value less than the known metabolic rate of the tissue, we may conclude that in this experiment asphyxia has been incomplete. If asphyxia is being produced, not by direct withdrawal of the oxygen supply to the preparation, but by a drug, such as cyanide, which inhibits the uptake of oxygen by the tissue, it must of

course be ascertained that the drug has not, in addition, a direct action on the rate of utilization of energy-producing material, i.e. the metabolic rate of the tissue, for a similar change in the slope of the line would occur if the drug produced complete inhibition of oxygen uptake together with a reduction in the resting metabolic rate. Since, however, by this method, the resting metabolic rate is determined only in relation to the metabolic rate of the muscle beating fifteen times per minute, the slope of the line would only become steeper if the resting metabolic rate were reduced to a greater extent than that of active muscle. Such an action, specific to resting as opposed to active muscle, is however unknown; in fact the reverse is usually found, namely an inhibition of active metabolism, the resting metabolism being unaffected [Clark, 1935*b*]. The only drug known to reduce the resting as well as the active metabolism is acetylcholine, which while abolishing contraction completely, reduces the resting metabolism by one-half [Clark, 1935*b*]. It therefore seems unlikely that cyanide which, in the normal muscle, only produces a slight reduction in the contractions comparable with that produced by pure oxygen lack, will have an effect on the resting metabolism of sufficient magnitude to alter the slope of the line.

The results of the experiments in which cyanide was used are shown plotted in Fig. 3. The concentrations used were *M*/150, *M*/500, *M*/1000 and *M*/4000. In each experiment the values for the rate of fall of the mechanical response at the various rates of beating were expressed as percentages of the value at 15 per min. It was then found that the points obtained from the experiments with *M*/150, *M*/500 and *M*/1000 cyanide all fell on the same line which cut the ordinate at a point corresponding to a resting metabolic rate of 8 p.c. of that of the strip beating at 15 per min. This value is low compared with the resting metabolic rate of the ventricle, but there remained the possibility that this might be the correct value for the resting metabolic rate of the auricle. To decide this point two experiments were carried out in which the relation between rate of beating and rate of fall of the mechanical response was determined for complete asphyxia produced by bubbling nitrogen. The results of these experiments are also plotted in Fig. 3, and although the points are somewhat scattered, there is no doubt that a line drawn through them cuts the ordinate at a point corresponding to a value considerably greater than 8 p.c., and we must therefore conclude that even the strongest concentration of cyanide used, *M*/150, did not completely abolish the uptake of oxygen by the strip. The third curve of Fig. 3 corresponds to *M*/4000 cyanide, and it is seen that this does not

meet the ordinate at any positive value, but cuts the abscissa at a point corresponding to a rate of 1 per min. It is to be concluded that at this rate of beating *M*/4000 cyanide will never reduce the mechanical response to half its normal value.

The results obtained with the higher concentrations of cyanide are of some importance in view of the controversy between the school of

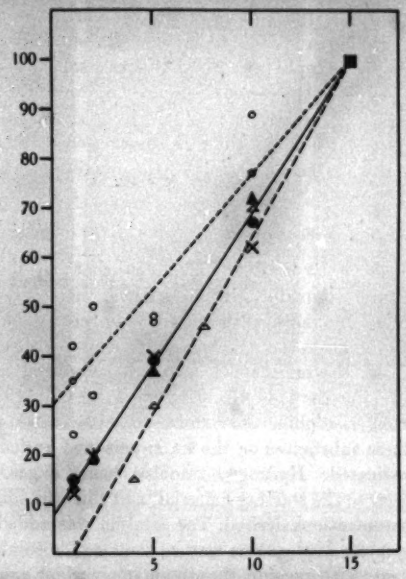

Fig. 3. Relation between rate of beating and rate of asphyxiation of I.A.A.-poisoned frog's auricular strip. Ordinate, 100/Ta; abscissa, rate per min. o - - - o, asphyxiation with N_2; •——•, ▲——▲, × - - - ×, treatment with *M*/150, *M*/500 and *M*/1000 NaCN respectively; △ - - - △, treatment with *M*/4000 NaCN.

Warburg on the one hand and Dixon and his co-workers on the other, regarding the action of cyanide on the oxygen consumption of isolated tissues. While Dixon and his colleagues have always found that the oxygen uptake of minced or sliced tissues is never completely abolished by cyanide, Warburg and his pupils have held that cyanide completely inhibits the uptake of oxygen by sliced tissues, and have attributed the

incomplete inhibition observed by Dixon to faulty technique, such as damage to the tissue by mincing and immersion in a fluid likely to harm it, i.e. phosphate buffer [Warburg, 1931]. In the present experiments there can be no doubt as to the good condition of the tissue, for, as mentioned earlier, the preparation will remain active for many hours, and we may therefore conclude that in this tissue there is a fraction of the normal oxygen uptake which is unaffected by cyanide.

Van Heyningen [1935] has recently compared the respiration of tissue slices suspended in phosphate buffer with that of slices of the same tissue suspended in phosphate-buffered Ringer's solution, and observed the effect of cyanide on the respiration under both conditions. He finds that the reduction of respiration produced by a given concentration of cyanide, though greater in the Ringer's solution than in the phosphate buffer, is nevertheless never complete, the maximum observed being about 90 p.c.

Effect of other oxidase poisons on the auricle

It has been shown by Keilin [1929, 1933] that the effect of cyanide on tissue respiration is mainly, if not entirely, the result of inhibition by the cyanide of an oxidase system known as the indophenol oxidase. If the effect of cyanide on the I.A.A.-poisoned heart is also a result of this oxidase inhibition, the same effect should be produced by other substances which inhibit the indophenol oxidase. Keilin [1933, 1936] has found that hydrogen sulphide and sodium azide both act in this way, and the effect of these substances on the I.A.A.-poisoned auricular strip was therefore investigated. Hydrogen sulphide being a gas, a saturated solution was used as the starting material, and this was suitably diluted to give the concentrations desired. The solution was added to the bath from a micro-pipette in the same way as the cyanide solutions, and, as in the experiments with cyanide, the stream of air which supplied oxygen to the strip was passed on its way to the bath through a solution of hydrogen sulphide of the concentration under investigation. The sodium azide, being non-volatile, was simply added to the bath from the micro-pipette.

Preliminary observations were made on the unpoisoned strip to determine whether either of these substances had any toxic action which might be confused with the effect which was expected to result from inhibition of the oxidase. It was found, however, that both substances in a concentration of $M/100$ produced no more than a slight and gradual fall in the mechanical response, similar to that produced in the

unpoisoned heart by simple asphyxia or cyanide (Fig. 4). The strip was then poisoned with I.A.A. and the substances tested again. Both, in a concentration of *M*/100, produced an almost immediate and rapid fall in the mechanical response terminating in contracture, an effect exactly similar to that of cyanide or simple asphyxia (Fig. 4).

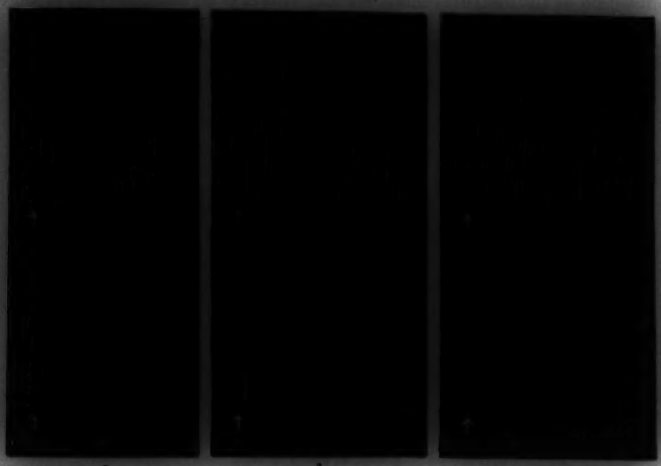

Fig. 4. Action of NaCN, H_2S and NaN_3 on normal (upper tracings) and I.A.A.-poisoned (lower tracings) frog's auricular strip. Drug in bath at arrow in each case: *a*, *M*/500 NaCN; *b*, *M*/100 H_2S; *c*, *M*/100 NaN_3. Time in min.

According to Keilin the function of the indophenol oxidase in the cell is to oxidize cytochrome, which then acts as a hydrogen acceptor in the dehydrogenation of metabolites which have been activated by a dehydrogenase. Keilin gives the following diagram to illustrate this:

$$\text{Dehydrogenase} + \text{Substrate} \left\langle \begin{matrix} H \\ H \end{matrix} \right. \rightarrow \text{Cytochrome } O\text{-Oxidase} + O_2$$

Narcotics

KCN
H_2S
NaN_3

Since cardiac muscle can work anaerobically as well as aerobically, we must conclude that under anaerobic conditions some other cell constituents, probably products of metabolism, replace the oxidized cytochrome as hydrogen acceptor. Further, since the heart poisoned

with I.A.A. fails in the absence of oxygen or as a result of treatment with substances which inhibit the indophenol oxidase, we must conclude that I.A.A. prevents the replacement of oxidized cytochrome by other substances in its function as hydrogen acceptor.

If we now turn to the other side of the picture and consider the function of the dehydrogenase, we find that it is an essential part of the process under all conditions, aerobic and anaerobic, in normal and in I.A.A.-poisoned muscle. We should therefore expect that inhibition of the dehydrogenase would cause failure of the heart under all the above conditions. This was tested on the auricular strip using ethyl urethane as the dehydrogenase inhibitor. The concentration of ethyl urethane used was 0·3 *M* and it was tested first on the normal unpoisoned strip and then

a *b*

Fig. 5. Action of 0·3 *M* ethyl urethane on *a*, normal and *b*, I.A.A.-poisoned frog's auricular strip. Ethyl urethane in bath at arrow in each case. Time in min.

on the same strip after poisoning with I.A.A. The effect was found to be the same in both cases, namely an immediate and fairly rapid fall in the mechanical response ending in complete disappearance of contraction, the strip remaining relaxed (Fig. 5). On washing out the drug the strip rapidly recovered. The results of these experiments do not agree with the hypothesis that the contracture of muscle, both cardiac and skeletal, which is the final effect of asphyxia after I.A.A.-poisoning, is the result of exhaustion of all sources of energy for contraction. Lundsgaard [1929], in his original work on lactic acid-free contraction, found that muscle in contracture contained no phosphagen, and concluded that the breakdown of phosphagen was the ultimate source of energy for contraction, the function of the breakdown of carbohydrate to lactic acid being to provide energy for the reformation of phosphagen. When the breakdown of carbohydrate was stopped by asphyxiating the I.A.A.-poisoned muscle, contraction could continue only as long as

phosphagen was present, the contractions becoming smaller as the store of phosphagen was depleted, and when the phosphagen was finally exhausted the muscle failed completely and went into contracture. More recent work however has shown that phosphagen cannot be the ultimate source of energy for contraction, for under suitable conditions asphyxiation of the I.A.A.-poisoned heart will cause it to fail and go into contracture with a large proportion of its phosphagen intact [Clark & Eggleton, 1936]. The results of the present investigation throw further doubt on the hypothesis for the following reason. If contracture is really the result of the exhaustion of all sources of energy, it should occur whenever these sources are cut off and by whatever means. One means of cutting them off is asphyxia after I.A.A. poisoning, and another, as shown above, is poisoning with ethyl urethane which inhibits the dehydrogenation processes essential in the breakdown of metabolites. Application of either of these methods causes complete failure of the contractions, presumably on account of depletion of the ultimate source of energy, yet when the method used is asphyxia after I.A.A. poisoning this failure is followed by contracture, while when ethyl urethane is applied the muscle fails in a state of permanent relaxation. It seems likely from this result that the contracture produced by asphyxiation of the I.A.A.-poisoned muscle is caused by the accumulation of some intermediate product in the breakdown of carbohydrates, since I.A.A. is known to act at a point at which partial breakdown has already taken place. A similar suggestion for the cause of contracture has been made by Ledebur [1934], who found that treatment of muscles with methyl glyoxal caused them to go into contracture.

Attempt to augment the cyanide insensitive fraction

It has been suggested [see Wagner-Jauregg, 1935] that the fraction of cell respiration which is insensitive to cyanide is carried on with the autoxidisable "yellow oxidation catalyst" of Warburg & Christian [1932] as hydrogen acceptor. It seemed possible that the addition of this, or another substance of a similar nature, to the frog's auricle poisoned with I.A.A. might increase the cyanide insensitive fraction of the respiration to such an extent that a dose of cyanide which ordinarily produced complete abolition of the contractions would now only produce a partial inhibition. A supply of the "yellow oxidation catalyst" was not available but a sample of lactoflavin,[1] the autoxidisable constituent

[1] I am indebted to Messrs Hoffmann La Roche for a sample of this substance.

of this substance, was obtained and tested but with negative results. Various concentrations were tried and it was found that concentrations of 1 in 40,000 and 1 in 20,000 had a definite toxic effect, producing a rise of the base line resulting from incomplete relaxation. A 1 in 60,000 concentration only produced this effect after longer contact with the auricle and this concentration was used in the experiments with cyanide. The procedure was as follows. The auricle was poisoned with I.A.A. and a record taken of the effect of the dose of cyanide to be tested ($M/500$). Lactoflavin was then added to the Ringer's solution and allowed to act for 20–30 min. and the same dose of cyanide was tested again. The cyanide then still produced complete abolition of contraction and, further, the rate of fall of the mechanical response was no different from that observed in the control observation without lactoflavin. This negative result was not entirely unexpected since it is stated by Wagner-Jauregg [1935] that lactoflavin does not act as hydrogen acceptor in the dehydrogenation of succinic acid by frog muscle although the latter contains quite a high proportion of succinode hydrase and readily dehydrogenates succinic acid in presence of methylene blue.

Summary

1. Cyanide, in sufficient concentration, has an action similar to that of complete oxygen lack on the frog's auricle poisoned with iodo-acetic acid.

2. It is shown by an indirect method that the oxygen uptake of the frog's auricle is not completely abolished by concentrations of cyanide up to $M/150$.

3. Other substances (H_2S, NaN_3) which, like cyanide, are oxidase poisons, have the same effect as cyanide on the auricle poisoned with iodo-acetic acid.

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THE ABSORPTION SPECTRA OF VISUAL PURPLE AND OF INDICATOR YELLOW

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It was stated by Kühne [1878] that on exposure to light a freshly dissected retina or a solution of visual purple becomes orange, chamois, and pale yellow in turn before finally becoming colourless. From Kühne's descriptions it is clear that the whole sequence was not always observed, but he applied the term "visual yellow" or xanthopsin to the intermediate substances in general. He called the colourless phase "visual white" or leucopsin, and it seems that he was led to give a name to this invisible product on the grounds that these colourless solutions gave a green fluorescence not originally present.

Köttgen & Abelsdorff [1896], who made a careful study of the absorption of visual purple at different wave-lengths, were unable to observe the presence of visual yellow. They found that there was a progressive decrease in the density of solutions which had been exposed to light for increasing times, and that there was no increase in density at the blue end of the spectrum to indicate the appearance of a freshly formed yellow substance. This result was confirmed by Trendelenburg [1904]. Garten [1906], however, obtained clear evidence of the formation of a yellow breakdown product. Many other papers have been published on visual yellow, and on the whole the evidence has been in favour of the formation of this substance when visual purple is bleached. It might seem a simple matter to decide the issue, but there are complicating factors which make observations difficult.

Holm [1922], from visual observation of the bleaching of intact retinæ, came to the conclusion that visual yellow appeared only when high illuminations were used. He thought that visual yellow acts as a

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colour filter to protect the eye against the effects of very high illuminations. There is, however, an important factor which explains why Holm and others have observed visual yellow under some conditions and not under others. It is probable that a substance with a deep yellowish colour is nearly always formed when visual purple is bleached by light, but that it is decomposed thermally at a rather rapid rate. If, therefore, the illumination is high, the unstable intermediate substance has not had time to decompose before all the visual purple has been bleached, but if the illumination is low the first-formed intermediate substance will have decomposed before the last of the visual purple has disappeared. The yellow is masked by the purple colour.

There are most probably two distinct phases in the fading of Kühne's "visual yellow": their presence can be demonstrated by a simple experiment. Two samples of visual purple, one of which had been cooled in ice and the other warmed to about 30° C., were brought into daylight. The warmed solution after bleaching was a pale yellow; the cooled solution, however, was a deep vermilion-orange. The orange colour lasted for a considerable time at 0° C., but on warming the solution it became pale yellow. It is proposed to use the terms "transient orange" and "indicator yellow" to describe the two phases, both of which are embraced by Kühne's term "visual yellow". Later in this paper further evidence for the separate existence of transient orange will be given.

Owing to the fleeting appearance of transient orange at room temperatures its existence has apparently not been suspected by many authors, and they apply the term "visual yellow" to the comparatively stable yellow substance which is left after visual purple has been exposed to light for some minutes. The experimental details given in the papers of Garten [1906] and others make it fairly certain that these authors were dealing with the substance which is here called indicator yellow. The reactions of this latter substance provide the clue to the fact that some workers have been unable to demonstrate the formation of any yellow substance when visual purple is bleached.

It was suggested by Krause [1934] and Chase [1936] that "visual yellow" is an acid-base indicator. Nakashima [1929] had shown that "visual yellow" is a deeper yellow in acid than in alkaline solutions, and Chase published curves to show that the course of bleaching of visual purple is not the same in acid as it is in alkaline solutions. In solutions buffered between *p*H 5·8 and 7·0 there was an increase in density at the blue end of the spectrum on bleaching, showing that a yellow substance had been freshly formed. Chase did not observe an increase in density

in the alkaline solution (*p*H 9·3) but his readings were not taken at wavelengths shorter than 430 mμ, whereas results quoted in the present paper make it clear that an increase in density would not be expected at wavelengths longer than 420 *mμ*. Furthermore, it was shown by Chase that partially bleached alkaline solutions developed the yellow colour if they were made acid in the dark room and that the final yellow colour of an acid solution disappeared when alkali was added. Chase's observations are of great importance, and as pointed out by him and earlier by Nakashima this reaction may explain the discrepancy between the results of Garten and Köttgen & Abelsdorff.

Simple unpurified extracts of visual purple contain unstable yellow impurities, and their presence will obviously complicate the detection of a newly formed yellow substance. At 430 mμ in a simple unbleached solution (*p*H 7·0) of density 1·30, visual purple has a density of about 0·31 and the impurities a density of 0·99. The total density of the bleached solution is about 1·33, to which impurities as before contribute 0·99 and the indicator yellow a density of 0·34. The density of the impurities is about three times that of the indicator yellow, and the net increase in density on bleaching only about 0·03. It would not be surprising if the small net increase in density were missed whether the examination were made by the naked eye or spectroscopically.

Wald [1936] has also confirmed that the intact retina first bleaches to an orange colour when exposed to light, and that in time the orange is replaced by a yellow substance giving the typical indicator reactions. In a series of papers Wald [1935, etc.] describes the extraction of a carotenoid pigment from freshly bleached retinæ which he calls retinene. When retinæ have been bleached and allowed to stand they yield a substance which appears to be vitamin A. Both extractions are made with petrol ether. Wald suggests that his retinene is Kühne's "visual yellow" and that "visual white" is vitamin A. I have found that transient orange and indicator yellow in aqueous solution are not extracted by petrol ether; it would appear that these substances are neither retinene nor vitamin A and the purely descriptive terms of "transient orange" and "indicator yellow" are to be preferred.

Solutions of visual purple are difficult to prepare and their spectroscopic examination is not easy owing to their great sensitivity to light. The object of the present experiments was to obtain reliable absorption curves in the visible spectrum both for visual purple and for indicator yellow. The experiments were made with solutions buffered to different hydrogen-ion concentrations with a view to determining the degree of

dissociation of visual purple and its derivatives. This information was primarily needed in connexion with some work on the photochemical bleaching of visual purple [Dartnall *et al.* 1936], but the results obtained may also be of importance in the explanation of visual sensation, and in the solution of the problem of the chemical constitution of visual purple.

Preparation of solutions

Solutions of visual purple may be prepared by placing the dissected retina in a solution of bile salts [Kühne, 1878], of digitonin [Tansley, 1931] or in one of a variety of other substances having hæmolytic properties [Hosoya, 1933]. After half an hour or so the envelopes of the rods will have ruptured, liberating the visual purple. The mass is then centrifuged and the supernatant fluid will contain the visual purple in solution. Such a solution is usually coloured an orange red and never reddish purple as would be the case if it were pure. Spectroscopic examination shows that these solutions absorb a very large proportion of the shorter wave-lengths (Fig. 1, top curve), and the density curves published by some authors even show an increasing absorption from the red to the blue end of the spectrum with a slight unevenness in the curve at the wave-length of maximum absorption (502 mμ) of the visual purple: the density of a pure solution should decrease progressively from 502 mμ towards both ends of the spectrum. Very little improvement can be made by the choice of solvent [Hosoya & Bayerl, 1933], and there is no doubt that there are yellow substances present in the extracts which account for the absorption of the shorter wave-lengths. Simple extractions of the whole retina are usually opalescent, and although the suspended matter cannot be removed by the centrifuge, oftener than not it is precipitated in an hour or so after preparation. The presence of yellow impurities although undesirable would not prevent accurate quantitative photometric work if they were stable, but they are not, and if one measures the wave-length absorption at intervals throughout the day it is found that considerable changes are taking place in the solutions. Something can be done to stabilize the solutions by centrifuging them for as long as 2 hours [Chase, 1936] and by attention to other details [Hosoya & Saito, 1935], but even so it is probable that the changes in the yellow impurities are of the same order as those produced by the formation of "visual yellow". Kühne [1895] described a method of purification involving the precipitation of visual purple by saturation with magnesium sulphate, but I could not repeat the result. I have since found that

visual purple is precipitated by full saturation with sodium sulphate but only if the temperature is raised to 30° C.

A new method has been devised for the preparation of visual purple solutions which are free from opalescence under most conditions, contain only a small amount of yellow impurities and which are stable for many hours at room temperature. In this new method the first step is the preparation of a suspension of retinal rods; the second step is the extraction of lipoids, etc., from the desiccated rods by petrol ether; in

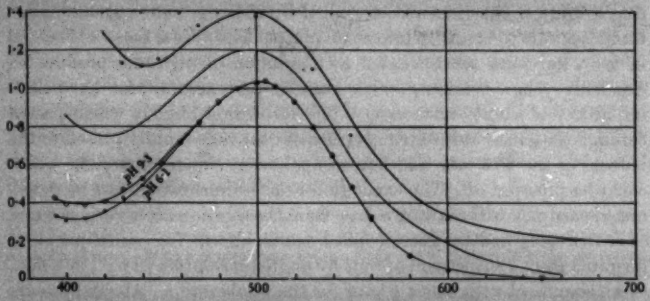

Fig. 1. Absorption curves of unbleached retinal extracts showing the elimination of impurities at different stages of purification. Values corrected so that the density of the contained visual purple at 502 mμ is 1·00 exclusive of impurities. Top curve: Extraction of whole retina with 2 p.c. digitonin solution. Extraction with 4 p.c. bile salts gives values shown by the points [Chase, 1936]. Middle curve: A suspension of "rods" was given a preliminary washing with a *p*H 4·6 buffer solution. Lower curve: Extracts used in present paper in which rods were also washed with petrol ether. Final solutions buffered to *p*H 6·1 (•—•) and *p*H 9·3 (○—○). The same preparation was used differing only in the added buffer. Abscisse: wave-length (mμ). Ordinate: density.

the third step some of the proteins are rendered insoluble by treating the rods with an acid solution of *p*H 4·6, whilst finally the visual purple is extracted by a solution of digitonin.

The retina can rarely be removed from a frog's eye without a certain amount of pigment epithelium even after the animal has been very thoroughly adapted to darkness. An attempt was made to wash off the adhering pigment by gently rocking the retinæ in 0·6 p.c. NaCl solution. It was soon found that nearly all the rods were detached from the retina by this process. Advantage has been taken of this fact to prepare a fairly pure suspension of rods, free from the rest of the retina. The retinæ after

dissection were placed in a small test-tube about three-quarters filled with a 0·6 p.c. NaCl solution. The test-tube was then shaken vigorously and the contents poured into a filtering cone made of fine wire gauze (200 threads to the inch). The rods, pigment granules and a few isolated cells passed through freely, but the main mass of the nuclear and fibre layers of the retina were held back. The retained mass, which was usually quite colourless, was examined histologically by Dr Tansley. She found that the outer limbs of the rods were almost completely removed, and about half the inner limbs were left adhering to the nuclear and fibre layers. The frog's retina is not otherwise disrupted by shaking, but the mammalian retina appears to break up into small pieces. The retinal mass left behind in the wire gauze when treated by a solution of digitonin produced a decidedly yellow solution, which must in part account for the yellow impurities of simple extractions. The suspension of rods which passed through the gauze was centrifuged lightly; the rods rapidly passed to the bottom of the test-tube and left an opalescent supernatant fluid which could be pipetted off. The exact times for sedimentation can be determined easily by withdrawing a drop from the solution after various times of centrifuging and by microscopical examination. The visual purple is liberated from a suspension of rods by digitonin solution (2 p.c.) in 2 min. or so, compared with about ½ hour for the whole retina. About 1½ hours is necessary for complete extraction from the whole retina by 4 p.c. bile salts [Chase, 1936].

If the visual purple solutions obtained by a digitonin extract of the rods are mixed with a buffer solution of *p*H 4·6, there is a considerable precipitate. If this precipitate is allowed to form a sediment, it will be found to be coloured pink with visual purple, but some visual purple will remain in solution. It is probable, therefore, that the acid solution precipitates a protein on which some of the visual purple is adsorbed. Spectroscopic examination of the clear supernatant fluid obtained after centrifuging showed that there were less yellow impurities present and indeed the author saw a real purple tint for the first time in such a solution. To prevent the adsorption of visual purple on the precipitated protein the rods are first treated with the acid buffer and then with the digitonin solution: the residues after extraction are opaque and have scarcely any colour. The degree of improvement in the solutions can be seen in Fig. 1 (middle curve).

The solutions after the above treatment were still opalescent and absorbed an appreciable proportion of the red wave-lengths. It was thought that there were lipoids present in the solutions. The suspen-

sion of "rods" was therefore centrifuged and the debris dried in a vacuum desiccator. On the addition of petrol ether there was a moderate liberation of a yellow substance. The "rods" were again dried and then extracted with digitonin solution. The solutions showed a considerable improvement on any previously obtained, with less of the yellow impurities (Fig. 1, bottom curves): they were usually quite free from opalescence, and a solution which transmitted only 28 p.c. of the incident light at 500 mμ transmitted more than 99 p.c. at 650 mμ.

In spite of the precautions to obtain pure solutions of visual purple, it will be seen from Fig. 1 that the solutions still probably contain yellow impurities. It is likely that some of these impurities can be removed by dialysis, but the actual proof that this is so is rather difficult to obtain, and since the technique is already elaborate the additional complications were thought not to be worth while. It may be recorded, however, that probably no visual purple passed through a cellophane membrane which usually passes molecules with a molecular weight of less than 20,000. The digitonin passed through fairly quickly.

Details of preparation. All operations are carried out by the light of an ordinary ruby dark-room lamp. The frogs are kept in the dark overnight, decapitated and the heads washed and dried. With a pair of fine scissors a single cut is made right across the cornea, avoiding the iris. The head is then held in the left hand with the forefinger on the palate just behind the eye and the thumb in the corresponding position on the top of the head. On applying gentle pressure the lens is first extruded and removed, whilst still further pressure brings out the vitreous and finally the retina. The latter is then picked up with fine forceps and placed in a small test-tube containing 0·6 p.c. NaCl in water. The retina should come away whole and its shape can often be seen as it floats out in the saline solution. There is often no adhering pigment when using *Rana temporaria* but with *R. esculenta* the retina is usually not quite clean. After all the retinæ have been expressed, the test-tube containing them is shaken vigorously for 20 sec. and the contents poured into a filter cone made of fine wire gauze. The retinal residues are then shaken for 15 sec. with more 0·6 p.c. NaCl solution to remove any entangled rods. The suspension of rods which has passed through the filter is next centrifuged at about 800 r.p.m. for 1 min. and the supernatant suspension of pigment cells, etc. pipetted off. The remaining rods are treated with more saline, shaken and centrifuged again. The operation is repeated until the supernatant fluid is fairly clear and the rods are then heavily centrifuged to remove as much fluid as possible. The remaining mass, which should occupy a surprisingly small bulk, is spread over the sides of the test-tube and placed in a vacuum desiccator for about 8 hours. The dried mass is next washed with petrol ether (B.P. 40–60°) until no further yellow colour comes away. The petrol ether is then evaporated off and 0·6 p.c. NaCl solution is added to the test-tube and the latter kept on ice overnight. In the morning the saline solution is replaced by an acid buffer solution (*p*H 4·6), the mass of rods is broken up, shaken vigorously and after 2 min. the buffer is removed, the rods are washed and treated with 1 p.c. digitonin solution. The contents of the test-tube are stirred by a steel ball for 10 min. at low temperature and then centrifuged at about 4000 r.p.m. for 10 min. or so, the temperature being kept as low as possible to prevent destruction of visual purple. The supernatant fluid, which should be quite clear, is removed and stored on ice.

For ordinary estimations 1 c.c. of digitonin is used for twelve large *R. esculenta*. Dilution with an equal volume of buffer solution produces a concentration of visual purple which is quite sufficient for most purposes.

Solutions prepared in this way keep well if buffered between *p*H 6 and 9·3. Absorption curves were made on some unbleached solutions immediately after preparation and again after they had been kept at room temperature (20° C.) for 3 hours or so. During this period there was no change in density at any wave-length, showing that neither the visual purple nor the yellow impurities had undergone any change. One solution with a *p*H of about 9·3 was kept for 4 days at 0° C., and showed only a 1 p.c. loss of concentration as measured by its density. Weakly alkaline solutions kept on ice for several months retained their colour. Visual purple is destroyed slowly at *p*H 5·2 and 10·0, and very rapidly at *p*H's much less than 5 or more than 11.

Apparatus. All measurements of density were made on the apparatus described by Bayliss *et al.* [1936]. The basis of the method consists in passing a narrow spectral band from a monochromator through the unknown solution on to a photoelectric cell. The cell is illuminated for an accurately timed interval, and the current passing through it charges up one plate of a condenser to which a Lindemann electrometer is also connected. The E.M.F. which tends to develop on the electrometer is neutralized by electrostatic induction, an equal and opposite potential being applied to the other plate of the condenser. The applied E.M.F. is proportional to the light which has fallen on the photocell.

Apart from mechanical and optical improvements no important change was made in the original apparatus beyond the fitting of a KG7 photocell (G.E.C.), which permitted readings to be taken down to 395 mμ. This type of cell has its greatest sensitivity at 436 mμ for an equal-energy spectrum. When used with a prismatic monochromator and a tungsten lamp as light source, its apparent response is greatest at about 510 mμ, falling off gradually towards the ends of the spectrum. A single monochromator of the type used in these experiments passes at least 1 p.c. impure stray light. If, therefore, measurements are made at a wave-length where the photocell gives 1/100 its maximal response, it follows that the total response will be due to light of the wave-lengths set on the monochromator scale and to stray light in about equal parts. Careful attention was therefore given to the choice of auxiliary colour filters through which the light was passed before entering the monochromator. The filters finally selected were Corning R-P Ultra (395–410 mμ), Wratten 35 (420–440 mμ), Wratten 47 (450–470 mμ), Wratten

45 (480–490 mμ), Wratten 59+44 (500–510 mμ), Wratten 58 (520–560 mμ), Wratten 22 (580–650 mμ). The Corning Ultra and the Wratten 35 pass an amount of red light which is very obvious to the human eye but which has practically no effect on the KG7 photocell.

Throughout this investigation readings were taken in rotation, the first on the unknown solution, the second on the control consisting of a plain solution of digitonin. From the two measurements the density of the unknown solution could readily be calculated. Readings were always taken from the blue towards the red end of the spectrum. When two unknown solutions were being measured against a common control, 5 min. were necessary to take readings at 395 mμ; the range 400–450 mμ was covered in a further 15 min.; the range 460–500 mμ in 13 min.; and the range 510–600 mμ in 12 min. The wave-lengths at which readings were taken are given in the tables.

Spectroscopic investigations of visual purple have often been confined to a few scattered wave-lengths because the solutions were placed between the light source and the monochromator and consequently bleached rapidly. Repeated trials with the present apparatus have shown that the monochromatic patch produces no measurable bleaching during the course of an experiment. For instance, the monochromator was set at 505 mμ, near the maximal absorption of visual purple. After forty exposures the loss in concentration of the solution as measured by its density was less than 1 p.c. During the course of an experiment about twenty-four exposures are made, for the most part to wave-lengths which are much less potent than 505 mμ in bleaching visual purple.

The buffer solutions for the range *p*H 3–10 were those recommended by Clark and Lubs [Clark, 1928]. The *p*H 2 solution was that recommended by Sørensen and the *p*H 11 and 12 solutions those recommended by Kolthoff *et al.* [Clark, 1928]. The hydrogen-ion concentration of every extract was measured on a glass-electrode apparatus kindly lent by Dr P. M. T. Kerridge [1926]. Owing to the internal buffering of the solutions, the measured hydrogen-ion concentration was always nearer to neutrality than that of the added buffer. The shift was considerably less with the comparatively protein-free purified extracts than it was with simple extracts of the whole retina. If an equal volume of an *M*/20 buffer solution of *p*H 4·0 is added to an extract made from whole retinæ the colour (visual purple) is not destroyed, but with purified extracts made from rods the colour vanishes in a few seconds.

The apparatus used for the bleaching consisted of a 100 c.p. pointolite, the ball of which was focused by an *f* 3·8 lens on the cell containing the

solution. The image of the ball was 1·2 cm. in diameter, whilst the cell had a diameter of 1·0 cm. A monochromatic filter passing about 15 p.c. at its wave-length of maximum transmission (506 mμ) was placed in the beam of light. The bleaching of an alkaline solution was half completed in about 30 sec., and completed in about 6 min., but in order to ensure that all solutions were fully bleached an exposure of 8 min. was given.

With the exception of the values given in Fig. 1, all the quantitative results quoted in this paper are from five separate extractions. The same batch of *R. esculenta* was used in order to eliminate seasonal differences [Hecht & Chase, 1934] and the experiments were completed in a period of 3 weeks during July 1936.

Results

All results are given as densities. If the light incident on a solution is I_0 and the light transmitted is I_t, then the density, D, is given by the equation $D = \log_{10} I_0/I_t$. A solution which transmits 10 p.c. of the incident light has a density of 1, a solution which transmits 1 p.c. of the incident light has a density of 2.

If light passes through two solutions in contiguous cells, the total density is the sum of the densities measured separately. If one solution passes 10 p.c. and the other 1 p.c. of the incident light, the final transmission is 0·1 p.c. Expressed in densities the values are additive, density 1 + density 2 = density 3. Exactly the same applies to two substances present in one solution (Beer's Law), the final density is the sum of the densities of the components. In Fig. 2 the density of the unbleached solution at 410 mμ was 0·380 of which the visual purple contributed a density of 0·103 and yellow impurities contributed a density of 0·277. In the bleached solution the density was 0·546. Assuming that the yellow impurities remained unchanged, the density of the newly formed indicator yellow was 0·546 − 0·277 = 0·269.

The density (D) of a solution can be expressed in the form

$$I_t = I_0\, 10^{-D}.$$

In dealing with substances of known composition it is better to use the equation $I_t = I_0 e^{-\alpha c l}$ where α is the extinction coefficient per chromophoric group, c is the number of chromophoric groups per c.c., l is the length of the optical cell, and e is the natural base of logarithms. In a paper by Dartnall *et al.* [1936] on the photochemical bleaching of visual purple, the value of α at 505 mμ was found to be about $4{\cdot}0 \times 10^{-17}$ cm.^{2} for *R. temporaria*. Later determinations have given the value $5{\cdot}0 \times 10^{-17}$ cm.^{2} for *R. esculenta*. It follows that α for other wave-lengths can be calculated by multiplying the density of visual purple at that wave-length by $5{\cdot}0 \times 10^{-17}$ cm.^{2}, and dividing by 0·995 (the density at 505 mμ). This conversion is valid for visual purple densities, but since it is not known how many chromophoric groups of indicator yellow are derived from one of visual purple, the conversion of densities into extinction coefficients

would not be valid for indicator yellow. For this reason the densities of the solutions measured in the present experiments are expressed by the simplest equation using D. If, however, one chromophoric group of visual purple gives rise to one chromophoric group of indicator yellow, then the conversion is also valid for the latter substance.

The absorption of visual purple. If, in solution, visual purple were dissociated in the region of its chromophoric group to give free H^+ or OH^- ions, then the absorption curves would vary with the hydrogen-ion concentration of the solution. The change would be revealed either by a shifting of the point of maximum absorption or by a loss in density. When the absorption curves at the different *p*H's were compared it was found that the curves coincided at all but the shorter wave-lengths. Fig. 1 (lower curves) shows the absorption curves from the same extract of visual purple, two samples of which had been buffered at *p*H 6·1 and 9·3. It will be seen that the wave-length of greatest absorption is the same in both solutions and that both curves are of the same height at the maximum and for the longer wave-lengths. Towards the shorter wave-lengths, however, the solution at *p*H 9·3 becomes progressively denser than that at *p*H 6·1. It is possible that visual purple has absorption bands at wave-lengths outside the visible spectrum which have some absorption at the shorter visible wave-lengths. The difference between the solutions at *p*H 6·1 and 9·3 cannot, however, be due to alterations in the density of the absorption band centred at 502 mμ, because if this were so the change would be greatest at this wave-length; an alteration in the position of the maximum of absorption would also produce a change in the density at 502 mμ. The probable explanation is that the yellow impurities show some changes with hydrogen-ion concentration, but we cannot exclude the possibility that the difference in absorption is due to changes in some other part of the visual purple molecule. It will be seen in Table I that there is a progressive decrease in density in unbleached solutions for the shorter wave-lengths as the solutions are made less alkaline. At *p*H 5·2, however, the solutions are slightly denser at all wave-lengths. This is due to a slight opalescence which disappears when the solution is made more acid or more alkaline. The differences in the densities of solutions containing equal amounts of visual purple at various *p*H's, although slight, are of some importance in calculating the true absorption curves of indicator yellow. In the calculations of the absorption curves of indicator yellow advantage is taken of the fact that the relevant part of the visual purple molecule is undissociated and that the absorption curve centred at 502 mμ is unaffected by *p*H.

Fig. 2 shows the absorption curves for an unbleached extract and for the bleached solution after it had attained equilibrium. It will be apparent that there has been an increase in density at the blue end of the spectrum on bleaching, showing that a new substance has made its appearance. One cannot determine the absorption curve of visual purple itself by subtracting the bleached from the unbleached values because negative values would be obtained at the short wave-lengths. Unless one has a quite pure solution of visual purple it is necessary to make certain assumptions before its absorption curve can be determined. From what

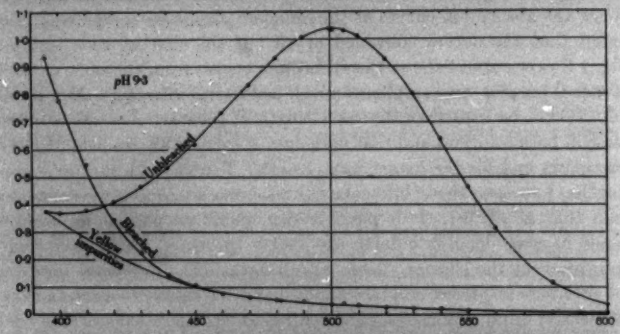

Fig. 2. Absorption curves of unbleached and bleached visual purple for one of the extracts at *p*H 9·3. The increase in density for the short wave-lengths on bleaching denotes the formation of indicator yellow. The dotted line shows the conjectural density of yellow impurities. Density of contained visual purple at 502 mμ = 1·00. Abscissæ: wave-length (mμ). Ordinate: density.

has been said under "Method of extraction" it will be clear that with successive purifications the density of the unbleached extract becomes progressively less at any one of the short wave-lengths (Fig. 1), and that with a quite pure extract it would probably be very small in the region of 400 mμ. The present solutions almost certainly contain yellow impurities, and before one can derive values for the density of visual purple at the shorter wave-lengths, it is necessary to assume values for the absorption of these impurities. If, as seems likely, the yellow impurities remain unchanged during bleaching, it would be impossible for them to have a density greater than the measured density of either the bleached or the unbleached solution at any wave-length. It is also likely that the impurities will behave similarly to the great majority of yellow biological

substances and will show a gradually increasing absorption towards the shorter wave-lengths. If the latter assumption is true then the absorption cannot be greater than that shown by the dotted line in Fig. 2. It is also probable that the absorption is not much less for the following reasons. In the first place the curve of the bleached solution rises rather abruptly from 450 mμ and becomes progressively higher as far as 395 mμ. It is probable that we are dealing with the edge of the absorption band of indicator yellow and that its absorption is very small at 460 mμ and almost negligible at 480 mμ. In the second place, it is likely that the density of visual purple decreases progressively towards the shorter wave-lengths. In the middle of the absorption band there will be a considerable difference in density between two wave-lengths 10 mμ apart and this difference will be revealed in the absorption curve of an impure extract, provided the impurities have not got any strongly marked absorption bands at these wave-lengths. At the ends of the curve the absorption band of visual purple probably flattens out as the values become small. The fact that the densities at 410 mμ were only a little greater than at 400 mμ probably means that this is the region where the absorption curve of visual purple begins to flatten out, and in order to make the density of visual purple small in this region it is necessary to assume that the density of the impurities is great. It will be assumed, therefore, that the density of the bleached solution between 395 and 460 mμ follows the dotted line in Fig. 2; that the density of the bleached solution between 460 and 480 mμ is almost wholly due to yellow impurities and that at longer wave-lengths it is wholly due to these impurities.

In the present determination of the absorption curve of visual purple, solutions buffered to a *p*H of 9·3 were used because at this value indicator yellow gives almost its lowest density values in the visible spectrum. There is actually a little less visible indicator yellow at *p*H 10·0, but visual purple is not quite stable at this alkalinity.

It was known from preliminary work that the maximum of absorption of visual purple alone was between 500 and 505 mμ. The purity of the spectral bands for most experiments was about 1·2 at 436 mμ; about 1·8 at 496 mμ; 2·0 at 546 mμ and 2·5 at 579 mμ. A special run was made on one of the denser solutions to locate the maximum more accurately. The slits of the monochromator were narrowed to give a band of wave-lengths whose purity was 0·8 mμ and many measurements of density were made in the region of the maximum. It was possible to locate the maximum absorption at 502 mμ. Five solutions were available for the calculation of the absorption curve of visual purple. For each of the

solutions a graph was drawn giving the bleached and unbleached densities; the probable values for the yellow impurities were then drawn freehand in the same way as has been done in Fig. 2. The densities read off from the freehand curve were subtracted from the densities of the unbleached solutions for the range 395–480 mμ: for the range 490–650 mμ the experimentally found bleached values were used. The densities for visual purple so obtained were for solutions of varying strength made on different days: all values were then multiplied by a factor to make the density at 502 mμ equal to 1·000. These factors were subsequently used for comparing solutions at other hydrogen-ion concentrations. The figures obtained were averaged (Table I). There was good agreement between the five experiments.

There have been very few previous determinations of the absorption spectrum of visual purple, and all have relied in one form or another on the simple procedure of subtracting the values for the bleached solution from those of the unbleached solution. Since the absorption curves of the bleached solutions vary with hydrogen-ion concentration, it follows that the apparent maximum absorption of visual purple obtained by subtracting the bleached from the unbleached values will also vary with the *p*H of the solution. The error introduced is small, however, between *p*H 6·1 and 10·0. If the true maximum of absorption of visual purple is at 502 mμ, the apparent maximum obtained by subtraction with solutions of *p*H 6·1 is at about 506 mμ. Of the previous determinations that made by Köttgen & Abelsdorff [1896] is the most important. Their maximum absorption is at about the same wave-length (500 mμ) but otherwise the agreement is not good, their values for the longer wave-lengths being as much as 20 p.c. greater than ours, whilst those for the shorter wave-lengths are much less. Their cross-over wave-length (the significance of which will be discussed later) is at about 425 mμ, from which it is concluded that the *p*H of their solutions was about 8·0. This means that their solutions did contain some indicator yellow although they believed otherwise, and the derived absorption curve for visual purple is of the shape which would be expected. Hosoya & Saito's [1935] values are in even worse agreement. Their maximum of absorption is at 510 mμ and the absorption curve for visual purple is very low indeed for the shorter wave-lengths. The present author can explain their results only by assuming that their solutions were acid and that they worked at a low temperature. From Chase's results it is possible to calculate some points on the absorption curve. From the values at 470, 480, 500 and 530 mμ in solutions at *p*H 9·3, it is clear that the maximum absorption

TABLE I. Showing the experimental values (corrected for concentration) for the densities of the unbleached and bleached solutions at various pH's. The values at pH 9·3 are the averages of five determinations. The density of the visual purple at 502 mμ in each unbleached solution is 1·00; the density in excess of 1·00 is due to yellow impurities. The densities of visual purple itself at different wave-lengths are also shown; the values are the average of five extractions and the mean variations from the average are shown. Columns "pH 5·2 B. 1" and "B. 2" give respectively the densities of the bleached solutions taken as soon as possible after bleaching and 3 hours later at 20° C.

Wave-length (mμ)	Visual purple	Mean variation	pH 10·0		pH 9·3		pH 8·5		pH 7·7		pH 7·0		pH 6·1		pH 5·2		
			U.*	B.*	U.	B.	U.	B.	U.	B.	U.	B.	U.	B.	U.	B. 1	B. 2
395	0·001	—	0·38	0·90	0·39	0·93	0·37	0·93	0·34	0·95	0·34	0·88	0·33	0·82	0·39	0·75	0·58
400	0·016	—	0·38	0·75	0·38	0·79	0·38	0·82	0·34	0·81	0·32	0·79	0·30	0·77	0·37	0·73	0·54
410	0·103	—	0·37	0·51	0·39	0·55	0·38	0·57	0·35	0·63	0·34	0·69	0·32	0·71	0·39	0·76	0·48
420	0·196	—	0·41	0·31	0·42	0·35	0·40	0·37	0·38	0·48	0·38	0·57	0·35	0·61	0·43	0·76	0·43
430	0·306	—	0·46	0·19	0·47	0·23	0·45	0·25	0·44	0·37	0·44	0·48	0·42	0·54	0·49	0·74	0·39
440	0·416	—	0·54	0·12	0·54	0·15	0·51	0·17	0·51	0·30	0·52	0·40	0·49	0·47	0·57	0·72	0·35
450	0·530	—	0·63	0·09	0·62	0·11	0·61	0·14	0·61	0·23	0·63	0·34	0·60	0·39	0·66	0·66	0·31
460	0·661	—	0·74	0·08	0·74	0·09	0·72	0·10	0·73	0·20	0·73	0·29	0·71	0·33	0·77	0·57	0·27
470	0·780	±0·005	0·85	0·07	0·85	0·07	0·83	0·08	0·84	0·16	0·85	0·25	0·82	0·27	0·88	0·50	0·24
480	0·878	±0·005	0·94	0·07	0·94	0·06	0·93	0·07	0·93	0·14	0·94	0·21	0·92	0·23	0·98	0·41	0·21
490	0·953	±0·005	1·01	0·06	1·01	0·06	1·01	0·06	1·01	0·11	1·02	0·18	1·00	0·18	1·04	0·33	0·18
500	0·997	±0·004	1·04	0·04	1·05	0·05	1·04	0·05	1·04	0·09	1·04	0·15	1·03	0·14	1·07	0·25	0·15
505	0·993	±0·004	1·04	0·05	1·03	0·04	1·03	0·05	1·03	0·09	1·03	0·14	1·03	0·13	1·06	0·22	0·14
510	0·975	±0·002	1·02	0·04	1·02	0·04	1·01	0·04	1·01	0·08	1·01	0·13	1·01	0·12	1·04	0·19	0·13
520	0·899	±0·004	0·93	0·03	0·94	0·04	0·94	0·03	0·94	0·06	0·94	0·11	0·93	0·09	0·96	0·15	0·11
530	0·780	±0·006	0·81	0·04	0·81	0·03	0·80	0·03	0·82	0·05	0·81	0·08	0·80	0·07	0·84	0·12	0·10
540	0·622	±0·009	0·64	0·03	0·65	0·03	0·65	0·02	0·66	0·04	0·65	0·07	0·65	0·05	0·67	0·10	0·08
550	0·460	±0·008	0·48	0·03	0·48	0·02	0·49	0·02	0·49	0·03	0·48	0·05	0·47	0·04	0·50	0·08	0·07
560	0·310	±0·006	0·32	0·05	0·33	0·02	0·33	—	0·33	0·03	0·33	0·04	0·31	0·02	0·35	—	—
580	0·113	±0·006	0·13	0·02	0·13	0·02	0·12	0·01	0·13	0·01	0·13	0·01	0·12	0·01	0·15	0·05	0·05
600	0·034	±0·002	0·05	0·02	0·04	0·01	0·04	0·00	0·05	0·01	0·04	0·01	0·04	0·01	0·07	0·04	0·05
650	0·003	±0·004	0·01	0·01	0·01	0·01	0·00	0·00	0·01	0·01	0·00	0·01	0·00	0·00	0·03	0·03	0·03

* U. = Unbleached. B. = Bleached.

is not far from 500 mμ, and his values at these and other wave-lengths are scattered about the average curve obtained in the present experiment (given by the dotted line in Fig. 5).

The absorption curve of indicator yellow. The method of obtaining these curves for the *p*H range where visual purple is stable will first be described since the curves for values outside this range cannot be found with any accuracy. For one day's work not more than four samples each of 0·5 ml. of the unbuffered visual purple solution were accurately measured and to each sample 0·5 ml. of standard buffer was added. The accuracy of the dilution was checked by weighing. For each batch one

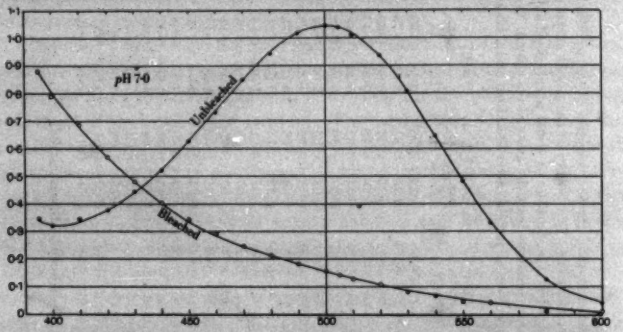

Fig. 3. Unbleached and bleached extracts at *p*H 7·0. Density of contained visual purple at 502 mμ = 1·00. Abscissæ: wave-length (mμ). Ordinate: density.

of the samples was buffered to *p*H 9·3 to act as a comparison standard between readings on different days. The two most acid samples were estimated immediately and the remaining two more alkaline samples were stored in a refrigerator and estimated later in the day. The absorption curve of the unbleached solution was first determined and the solution was then slowly bleached for a measured time in a constant illumination. The measurement of the absorption curve of the bleached solution began 10 min. after the beginning of bleaching and again after 3 hours at room temperature (19–21° C.). Some of the experimental results are given in Table I, and the values have been plotted for *p*H 9·3, 7·0 and 5·2 in Figs. 2, 3 and 4 respectively. A correction for concentration has been made by multiplying the densities by the factors mentioned on p. 344: at 502 mμ the density of the visual purple exclusive of impurities

is therefore 1·00. The formation of indicator yellow is shown by the increase in density at the short wave-lengths on bleaching. By comparing Figs. 2, 3 and 4 it will be seen that this increase is greater for the more acid solutions.

The density of the bleached solution is due partly to indicator yellow and partly to yellow impurities. Provided the absorption curve of these impurities is known, it is possible to find the density of the indicator yellow itself at any wave-length. It will be remembered that the densities of the yellow impurities themselves vary with hydrogen-ion concentration. It is therefore necessary to determine these densities for each sample. This was done by assuming that the visual purple content

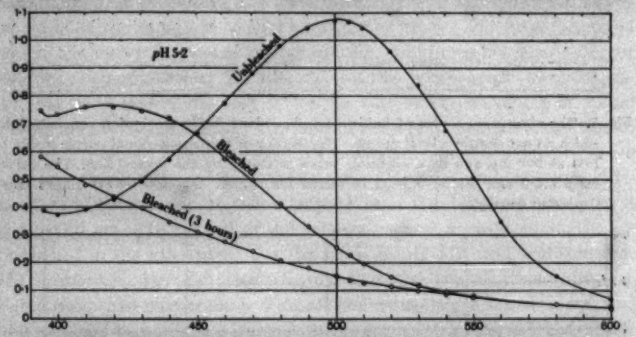

Fig. 4. Unbleached and bleached extracts at *p*H 5·2. The bleached solutions were estimated immediately after bleaching and again 3 hours later. Correction for concentration as in Figs. 2 and 3. Abscissæ: wave-length (mμ). Ordinate: density.

of the four samples was the same for each; the values for the yellow impurities were obtained by subtracting the density of the standard visual purple curve from the total density of the unbleached solution. An example will make this clear. At 520 mμ the density of visual purple itself was 0·90. For the extract at *p*H 7·0 the unbleached density at 520 mμ was 0·94; subtracting the visual purple density we obtain 0·04 for the density of the yellow impurities. The density of the bleached *p*H 7·0 solution at 520 mμ was 0·11; subtracting 0·04, the density of impurities, we obtain a value of 0·07 for the density of indicator yellow.

Fig. 5 gives the absorption curves of indicator yellow for the range *p*H 5·2–10·0. Most of the bleached solutions showed practically no loss

in density after standing at room temperature for 3 hours. The solution at *p*H 6·1 did, however, show a slight change and the solution at *p*H 5·2 showed a considerable loss of density with time (Figs. 4 and 6). It

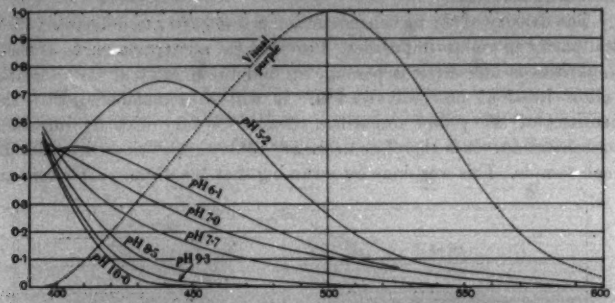

Fig. 5. The absorption curves of indicator yellow alone for hydrogen-ion concentrations which do not destroy visual purple. The density of the parent visual purple would be 1·00 at 502 mμ and its absorption curve is shown by the dotted line. The curves for *p*H 5·2 and 6·1 have been corrected for fading. Abscissæ: wave-length (mμ). Ordinate: density.

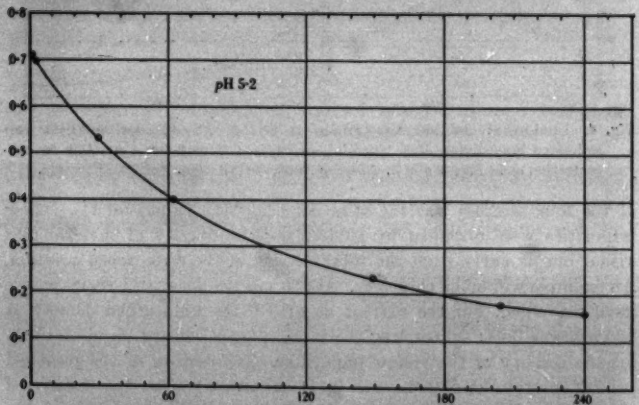

Fig. 6. The fading of indicator yellow with time in a solution buffered at *p*H 5·2. Bleaching was for 8 min. and the time intervals were measured from the end of bleaching. The density of the parent visual purple would be 1·00 at 502 mμ. Abscissæ: time (min.). Ordinate: density at 450 mμ.

follows that the readings taken late in a run will be relatively too low compared with those taken early. Readings on the bleached solutions at *p*H 5·2 and 6·1 were taken at 450 mμ immediately after bleaching and at regular intervals afterwards. On the assumption that a single substance was disappearing, the densities at the other wave-lengths were corrected to give the density at the end of bleaching.

The curves shown in Fig. 5 are very similar to the absorption curves of some ordinary laboratory indicators and there is a suggestion of an "iso-bestic" point at about 400 mμ. The absorption curve of visual purple is drawn on the same graph and for an equivalent concentration; that is to say, if a sample of visual purple had an absorption curve as drawn, then on bleaching, the absorption curve of the indicator yellow would be of the same size as that given in the figure. The wave-length at which the curve for visual purple crosses the curves for the indicator yellow is also of interest. We will call this the "cross-over" wave-length. At this wave-length the loss of density due to the bleaching of visual purple is exactly equalled by the gain in density due to the formation of indicator yellow. The cross-over wave-length is very constant between experiments on different days at the same *p*H value, and since it is independent of the amount of yellow impurities or the degree of bleaching it is a useful guide to the *p*H of the solutions whose absorption curves are published in the literature. For the more deeply coloured acid solutions the cross-over wave-length is longer than for the alkaline solutions. Some published curves show a cross-over wave-length between 490 and 500 mμ, and sometimes a second cross-over point in the red end of the spectrum. In our experience this result is always associated with intercurrent precipitation in the solutions measured.

Visual purple is destroyed by moderately strong acid or alkaline solutions, and it is not possible to produce accurate curves for indicator yellow in such solutions owing to the variations in the density of the yellow impurities with *p*H. The curves given in Fig. 7 for *p*H 10·8, 4·0 and 3·3 are probably a fair approximation. As before the comparable curve for visual purple is drawn. It will be seen that the curves taken at *p*H 4·0 and 3·3 are very similar to those at *p*H 5·2 (Fig. 5) and any differences which exist are possibly due to variations in the yellow impurities. The curves are, however, quite different for those taken in the range *p*H 10·0–6·1 and suggest that some tautomeric change has occurred. The height of the indicator yellow curve at *p*H 4·0 is not very different from the density of the visual purple from which it was derived. The colour of a solution at *p*H 3·3 is a deep chrome yellow.

The solutions were prepared by rapidly bleaching 0·5 ml. of visual purple solution and then adding 0·5 ml. of the appropriate buffer. The densities of the final solutions were estimated in the usual way with a comparison standard, buffered at *p*H 9·3. At *p*H 4·0 the indicator yellow was slowly decomposed but not quite so rapidly as it was at *p*H 5·2. A series of readings at 450 mμ was taken on the solutions at different times after bleaching. As with the solutions at *p*H 5·2 and 6·1, the readings at other wave-lengths were corrected on the assumption that a single substance was disappearing, the densities being calculated to give the values

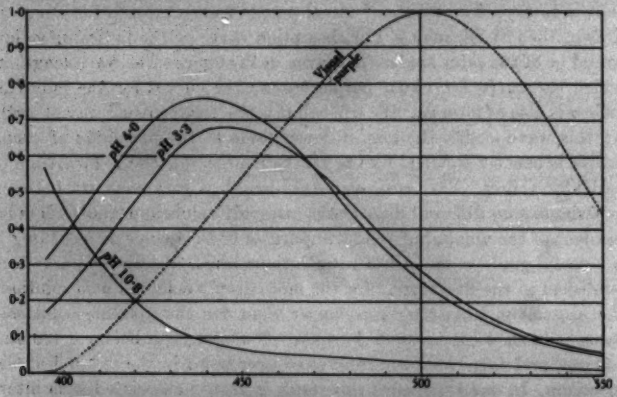

Fig. 7. The absorption curves for indicator yellow alone for hydrogen-ion concentrations which destroy visual purple. The density of parent visual purple would be 1·00 at 502 mμ and its absorption curve is shown by the dotted line. The curve for *p*H 4·0 has been corrected for fading. Abscissæ: wave-length (mμ). Ordinate: density.

immediately after bleaching. At *p*H 3·3 the indicator yellow is comparatively stable. The true values for the densities of indicator yellow were calculated as before by subtracting the densities of the yellow impurities from the values actually obtained. Since it is not possible to obtain an experimental value for the impurities at *p*H's where visual purple is decomposed, the values for the yellow impurities at the nearest *p*H on the same day's runs were used.

Nakashima [1929] found that when the colour of visual purple is destroyed by the addition of an acid, the solution becomes a deeper yellow than when an alkali is added. This observation was confirmed, and

it was shown in addition that the absorption curve of the indicator yellow is almost the same when the visual purple is "bleached" by acid as when an unbuffered solution is bleached by light and then buffered. Two 0·5 ml. samples of visual purple were taken: one was bleached by light and 0·5 ml. of buffer was added to bring the *p*H to 4·0; the other sample was bleached by the addition of 0·5 ml. of the same buffer solution in the dark room. The absorption curves (Table II) of the two solutions showed

TABLE II. Showing the experimental values (corrected for concentration) for the densities of bleached solutions at various *p*H's. As in Table I a solution in which the visual purple alone had a density of 1·00 at 502 mμ would bleach to give a solution with the densities given.

Wave-length	*p*H 10·8 bleached	*p*H 4·0 bleached by light	*p*H 4·0 bleached by buffer	*p*H 3·3 bleached
395	0·93	0·65	0·66	0·54
400	0·82	0·67	0·68	0·55
410	0·58	0·74	0·77	0·63
420	0·39	0·77	0·85	0·71
430	0·28	0·81	0·90	0·76
440	0·21	0·79	0·90	0·79
450	0·15	0·74	0·85	0·76
460	0·13	0·64	0·74	0·72
470	0·12	0·54	0·63	0·63
480	0·10	0·44	0·52	0·53
490	0·08	0·34	0·41	0·43
500	0·07	0·26	0·31	0·34
505	0·06	0·23	0·27	0·29
510	0·06	0·19	0·22	0·25
520	0·05	0·14	0·16	0·18
530	0·04	0·10	0·12	0·13
540	0·03	0·08	0·09	0·10
550	0·02	0·06	0·06	0·06
580	0·01	0·04	0·04	0·03
600	0·01	0·03	0·03	0·01
650	0·00	0·03	0·03	—

the same maximum and general shape of curve; in addition, the rate of loss of density was the same in both. Indicator yellow is probably the same both when visual purple is decomposed by light and when it is decomposed by the acid buffer. The density of the solution bleached by light was, however, slightly less; the difference is too great to be accounted for by experimental error.

The regeneration of visual purple. There is an increase in density of bleached solutions at *p*H 7·0 and 7·7 when they are kept in the dark for 3 hours. This increase is due to the regeneration of visual purple; it is greatest in the region of 500 mμ and has been a constant feature in experiments made during the last year. The increase would be accounted for if about 3 p.c. of the original visual purple were reformed. Hecht *et al.* [1936] have also noted the same phenomenon. It is stated that more than

half the increase in density is accomplished in the first 10 min. In the present work the reading at 500 mμ was not taken until about 30 min. after the end of bleaching—it is not surprising that Hecht's value (15 p.c.) is greater than ours. From earlier results got by the present author it is clear that regeneration is not confined to the *p*H range of 6·6–8·0; 10 p.c. regeneration has been repeatedly found at *p*H 9·3 (at 20° C.), but it appears to be a much slower process occupying about 24 hours. It is obvious that for this work one must use solutions which do not precipitate. Of rather greater interest than the mere phenomenon of regeneration is the fact that the density of the indicator yellow always shows a decrease when regeneration occurs. It is therefore probable that indicator yellow is taking part in the reformation of visual purple. The changes involved are, however, too small to admit of quantitative treatment. The cross-over wave-length, where the density of visual purple equals that of indicator yellow, remains much the same during regeneration.

Indicator yellow. By the use of the term "indicator yellow" it has been assumed that the substance formed from visual purple some little time after it has been bleached by light is an acid-base indicator. There are two reasons for this belief, first, that the densities of the bleached solutions vary with the hydrogen-ion concentration, the differences being often maintained for long periods. Secondly, that the changes are reversible. These reasons will be considered in more detail.

The yellow colour at *p*H 4·0, 5·2 and 6·1 is gradually lost and it is possible that more alkaline solutions are paler for the simple reason that more of the yellow substance has been broken down. If, however, the yellow substance were decomposed at an increasing rate when the solutions are more alkaline, it should be easy to detect the progressive loss of density with time at intermediate hydrogen-ion concentrations. Such a loss in density did not occur for the range *p*H 7·0–10·0 over a period of 3 hours. Furthermore, the acid solutions should remain the yellowest, whereas it was found that after 3 hours the measured density of the solution at *p*H 5·2 was less than that at *p*H 7·0. We are safe in assuming, therefore, that the end product of bleaching is stable between 7 and 11, but is unstable between *p*H 4·0 and 6·1. Hosoya [1933–4] who measured the loss of density with time in bleached solutions was probably investigating changes in yellow impurities as well as in what is here called indicator yellow. The changes can be followed in test-tubes by visual observation. In a series of bleached solutions at different *p*H's, the solutions at *p*H 10·0 and 9·3 are very pale lemon yellow. The depth of yellow increases gradually to *p*H 4·0 where it is very marked. The

solution at *p*H 3·3 has a slightly different reddish hue and is chrome yellow. If these solutions are kept at room temperature in sealed tubes for 24 hours or so, there is no appreciable change in most of the solutions, the solutions at *p*H 4·0 and 5·2 have, however, suffered a considerable loss in density and are the palest of all, being almost colourless.

The reversibility of the indicator change can be demonstrated quite readily in the test-tube. 0·5 ml. of a solution of visual purple was made

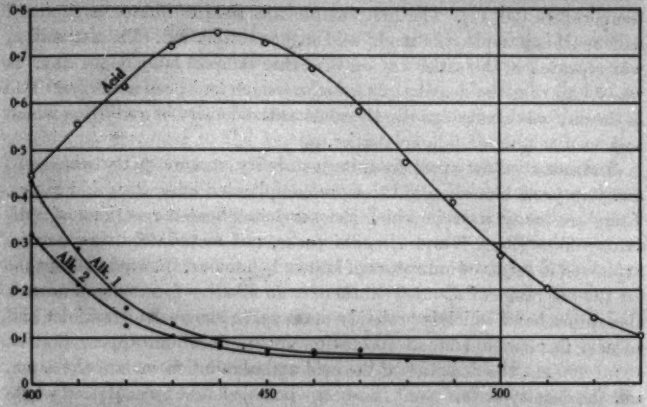

Fig. 8. The reversibility of the indicator yellow reaction. The absorption curve for an alkaline solution was first determined (Alk. 1). A curve was then taken on the acidulated solution (*p*H 5·0) and finally the solution was made alkaline again (*p*H 9·5) and another curve was taken (Alk. 2). Density of parent visual purple would be 1·00 at 502 mμ. Abscissæ: wave-length (mμ). Ordinate: density.

alkaline by the addition of an equal volume of 0·02 *N* NaOH. After bleaching the colour was pale lemon yellow. 0·1 ml. 0·2 *N* HCl was then added to make the solution acid; the colour became a deep lemon yellow, whilst on making the solution alkaline again the original pale yellow was restored. The change can be repeated any number of times. The absorption curves of these solutions were measured and the result is given in Fig. 8. The figures have been corrected for losses of density due to dilution and have been multiplied by a factor to make the density of the parent visual purple equal to 1·0. It will be seen that the density of the final alkaline solution (Alk. 2) is rather less than the density of the alkaline solution before the addition of acid (Alk. 1). The difference is

probably to be accounted for by the destruction of indicator yellow in the acid solution.

It has been shown that indicator yellow is unstable in solutions of certain acidity. In alkaline solutions, on the other hand, indicator yellow although scarcely visible to the eye, is quite stable and after several hours it is still capable of regaining its full yellow colour when it is acidulated. A solution of visual purple was made alkaline with 0·02 *N* NaOH bleached and divided into four samples which were stored at room temperature (20° C.). The first sample was then estimated as it stood and another sample was made acid before estimation. The estimation was repeated in the same way on the other samples later in the day. It was found that the density of a solution which had been acidulated late in the day was almost exactly the same as the density of a solution which had been acidulated 6 hours earlier.

Indicator yellow apart from its instability at some *p*H's behaves in many respects like some of the more complicated laboratory indicators. There are many ways in which the very complicated reactions of indicator yellow (Figs. 5 and 7) could occur, but fortunately they can be explained in terms of indicators of known behaviour. It would seem that for the *p*H range of about 6–10 there is an alkaline form with a narrow absorption band which is probably maximal in the near ultra-violet and an acid form with a broad absorption curve. It would appear that at about 400 mμ the densities of the acid and alkaline forms are the same, and the density at this point (iso-bestic point) should be unaltered whatever the relative proportions of the two forms. It is only the simplest indicators which have a well-defined iso-bestic point and the amount of scatter for indicator yellow is no greater than it is for an indicator such as phenol red. It can be shown theoretically that an indicator must pass from the almost purely acid form to the almost purely alkaline form in a range of about four *p*H units. If any further change in the absorption curve takes place outside this range it is due to a tautomeric change within the molecule. The densities of the indicator yellow for the range *p*H 10·0 to 6·1 do not conform to the standard equation relating *p*H and the degree of dissociation. The probable reason is that at *p*H 6·1 the molecule of indicator yellow has begun to undergo a tautomeric change. Changes of this nature are common in indicators, for example in thymol blue, and it appears that indicator yellow has a separate tautomeric form between *p*H 5·2 and 3·3. This range would, however, be very difficult to investigate quantitatively because of the thermal instability. It is a pity that the human eye is not sensitive to change of hue in the wave-

lengths being considered: if it were, the reactions of indicator yellow would form a striking colour display.

Transient orange. If two samples of alkaline visual purple, one of which has been cooled in ice and the other warmed, are placed in the light, the cooled solution bleaches to a vermilion-orange colour. On subsequent warming of the latter solution it loses its orange colour and becomes identical in colour with the solution which has been bleached in the warm. It seems probable that there is an intermediate phase in the bleaching of visual purple by light.

It has been noticed by Dartnall [1936] that during the bleaching of visual purple by monochromatic light, a "dark reaction" occurred. This dark reaction showed that when a partially bleached solution of visual purple was kept in the dark, there was a further loss of density roughly proportional to the amount of freshly bleached visual purple. The present author has made some preliminary experiments in which a sample of visual purple was bleached in a high illumination for about 15 sec., and the subsequent change of density was followed. It was found that at *p*H 7·0 and 9·0 there was an abrupt loss of density at 460 mμ due to the bleaching of visual purple followed by a further loss of density over a period of about 10 min. at *p*H 9·0 and 15 min. at *p*H 7·0. There appeared to be practically no change at 435 mμ (*p*H 7·0) and a slight gain in density at 410 mμ (*p*H 7·0). The changes in density are probably due to the same process as Dartnall's dark reaction and mark the breakdown of transient orange to the stable indicator yellow. Transient orange is apparently not formed in solutions at *p*H 5·2.

Discussion and conclusions

A tentative scheme for the bleaching of visual purple both by light and by moderately strong acids and alkalis is given in Fig. 9. The scheme has been drawn up merely to describe the facts recorded in this paper in a condensed form. According to the scheme when visual purple is bleached, transient orange is formed. The latter undergoes a thermal change leaving indicator yellow in the solution. Fairly strong acids and alkalis when added to visual purple form indicator yellow immediately. The reversible changes in the indicator yellow itself on altering the hydrogen-ion concentration appear to be extremely rapid. It is possible to reach the final indicator yellow stage at *p*H 4·0 by two routes, viz. either by adding acid buffer direct to a visual purple solution or by the action of light on a solution of about *p*H 6·5 and subsequent acidulation. There are also alternative routes to the indicator yellow of *p*H 11.

In view of the fact that indicator yellow behaves in all ways like an acid-base indicator such as thymol blue, it is possible that transient orange is a manifestation of indicator reactions. Most laboratory acid-base indicators take up their final colour very quickly, but there are exceptions of which China blue is an example. China blue may take some minutes before it comes to equilibrium in an acid or alkali solution and it is possible that the transient orange is behaving similarly.

It is well known that the staining reactions of the retina are changed after it has been exposed to light. The changes are difficult to interpret [Tansley, 1935], but most authors believe that the retina becomes acid

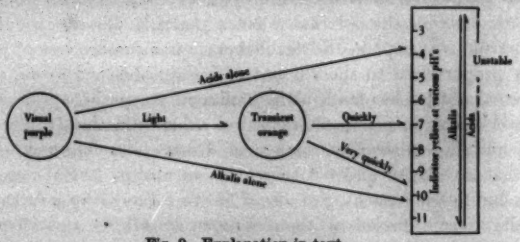

Fig. 9. Explanation in text.

when bleached. If this is true and if the reactions which occur in solutions of visual purple also occur in the intact retina, it means that the bleached whole retina must contain a yellow substance whose density is comparable with the visual purple which it has replaced. Considerable changes in the luminosity curve may be produced by these internal yellow filters.

Summary

1. A method is described for the preparation of reasonably pure solutions of visual purple which if buffered between *p*H 6·0 and 9·3 are quite stable at room temperature for several hours.

2. The absorption curves of visual purple extracts are unaffected by hydrogen-ion concentration except for small variations which can be explained by changes in the impurities present. If, therefore, the visual purple molecule is dissociated in solution, it is not in the region of its chromophoric group.

3. By making certain assumptions about the densities of impurities present, a fairly accurate absorption curve for visual purple has been obtained. The maximum absorption is at 502 mμ. The curve is different

from those obtained by many previous authors because their solutions were probably more acid than *p*H 9·0.

4. During the course of bleaching a well-defined intermediate substance is formed to which the name "transient orange" has been given. Transient orange is thermally decomposed to a yellow substance which has been named "indicator yellow".

5. Indicator yellow is pale yellow in alkaline solution, a deeper yellow of the same apparent hue in neutral solution and a deep chrome yellow in acid solution. Absorption curves for indicator yellow have been determined between 395 and 650 mμ over a range of hydrogen-ion concentration from *p*H 3·3 to 10·8. The accepted explanation of the results is that indicator yellow is an acid-base indicator, and for this belief fresh evidence has been produced. For the range *p*H 6·1 to 10·0 there is some indication of an "iso-bestic" point at about 400 mμ. The alkaline form at *p*H 10·0 has a narrow absorption band with a maximum which is probably in the near ultra-violet: the more acid form has a broad absorption band. Between *p*H 6·1 and 3·3 it appears that a tautomeric change takes place in the molecule and the absorption curves are quite different from those taken between *p*H 6·1 and 10·0. Indicator yellow is thermally unstable between *p*H 4·0 and 5·2 and slight changes can be found at *p*H 3·3 and 6·1. Between *p*H 7·0 and 10·8 it remains unchanged for several hours at 20° C.

As revealed by their absorption curves the reactions of indicator yellow are completely reversible.

6. Visual purple is stable between *p*H 5·2 and 10·0: outside this range it loses its colour and the indicator yellow formed is indistinguishable from that formed by first bleaching the solution in light and then adding the appropriate buffer.

7. A little regeneration of visual purple in bleached solutions occurs between *p*H 7·0 and 9·3 but only slowly in the more alkaline solutions.

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CHOLINESTERASE IN VOLUNTARY FROG'S MUSCLE

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Recent investigations by Dale, Feldberg and their collaborators have shown that impulses reaching motor nerve endings in voluntary muscle fibres cause a liberation of acetylcholine, and suggest that this substance may act as a transmitter of excitation from nerve to muscle. According to previous investigations [Chang & Gaddum, 1933; Plattner & Hintner, 1930], the amount of acetylcholine found in various tissues and the quantity of cholinesterase present can be brought into relation with the existence of the so-called cholinergic transmitter mechanism: the highest concentration of substrate and enzyme was—with a few unexplained exceptions—found in tissues whose main activity is controlled by nerves which are acting, or may be supposed to act, by liberating acetylcholine. As Brown *et al.* [1936] suggest, the acetylcholine liberated is more or less rapidly hydrolysed in order to enable the tissues to return to their previous condition, and therefore the distribution of the enzyme may vary with the kind of tissue. It was therefore of interest to obtain quantitative data of the rate of acetylcholine hydrolysis by voluntary muscle tissue.

Methods

The method developed for these experiments was based on the principle used by Ammon [1934] and Stedman & Stedman [1935]. These authors measured the rate of hydrolysis in blood serum and tissue extracts with a manometric method. In this method the formation of acetic acid formed during hydrolysis is measured by the liberation of CO_2 from bicarbonate added to the solution. The rate of the CO_2 output is proportional to the amount of enzyme present. By this means it is possible to compare the cholinesterase contents of different tissues.

We used a method which differs from that adopted by Ammon and by Stedman & Stedman in the different relation between enzyme and substrate. Amounts of acetylcholine between 0·2 and 0·6 mg. in 2–3 c.c. of total fluid are completely hydrolysed by 0·1 c.c. of horse serum in

60–100 min. The differences between two parallel determinations did not exceed 2–3 p.c. This method made it possible to examine quantitatively the course of acetylcholine hydrolysis in living tissue. Slices or pieces of tissue were suspended in a solution containing acetylcholine. After a suitable time the tissue was removed and an aliquot amount of the fluid taken for the manometric acetylcholine determination. In this way the remaining acetylcholine content was determined and subtracted from the acetylcholine content initially present. The difference represented the amount hydrolysed. Acetylcholine chloride of Hoffmann-La Roche was used in all experiments. All data are calculated for the neutral substance. To obtain the amount of base the figures must be multiplied by 0·8.

Results

Finely cut muscle of the frog

In this way the rate of hydrolysis of different concentrations of acetylcholine by frog's muscle (*Rana esculenta*) has been determined. Table I contains the results of experiments in which pieces of finely cut

Table I

No.	Weight of muscle tissue g.	Period of hydrolysis min.	Initial amount of ACh. mg.	ACh. remaining mg.	ACh. hydrolysed: Whole amount mg.	ACh. hydrolysed: By 100 mg. of tissue µg.	ACh. hydrolysed: Mean value µg.
1	0·400	50	0·906	0·635	0·271	68	66
2	0·410	50	0·906	0·565	0·341	83	
3	0·504	50	1·140	0·880	0·260	52	
4	0·455	50	1·140	0·857	0·283	63	
5	0·420	100	1·140	0·600	0·540	128	117
6	0·462	100	1·140	0·650	0·490	106	
7	0·487	165	1·140	0·375	0·765	157	148
8	0·393	165	1·140	0·590	0·550	140	
9	1·000	50	2·645	1·370	1·275	127	127
10	1·080	50	2·645	1·280	1·365	126·5	
11	0·670	100	2·645	1·240	1·405	210	209
12	0·700	100	2·645	1·190	1·455	208	
13	0·430	160	2·560	1·430	1·130	262	262
14	0·566	50	5·030	3·980	1·050	186	168
15	0·519	50	5·030	4·260	0·770	149	
16	0·594	100	5·030	3·180	1·850	312	281
17	0·587	100	5·030	3·560	1·470	250	
18	0·535	154	5·030	2·930	2·200	411	373
19	0·597	154	5·030	3·110	1·920	334	
20	2·10	50	14·625	10·950	3·675	175	184
21	2·05	50	14·625	10·700	3·925	192	
22	1·45	100	14·625	9·560	5·065	350	335
23	1·69	100	14·625	9·260	5·395	320	
24	1·22	160	14·625	7·820	6·805	558	544
25	1·19	160	14·625	8·330	6·295	529	

muscle from the upper half of the hindlegs were suspended in 5 c.c. of Ringer solution, containing varying amounts of acetylcholine. The Ringer solution used throughout these experiments contained in 1 litre 6·25 g. NaCl, 0·2 g. $CaCl_2$ and 0·15 g. KCl. To 100 c.c. of this solution 10 c.c. of a 1·1 p.c. $NaHCO_3$ solution were added. The experiments were carried out in Erlenmeyer flasks, a gas mixture containing 5 p.c. CO_2 and 95 p.c. N_2 being bubbled through the solution during the experiment to ensure a constancy of *p*H at 7·2.

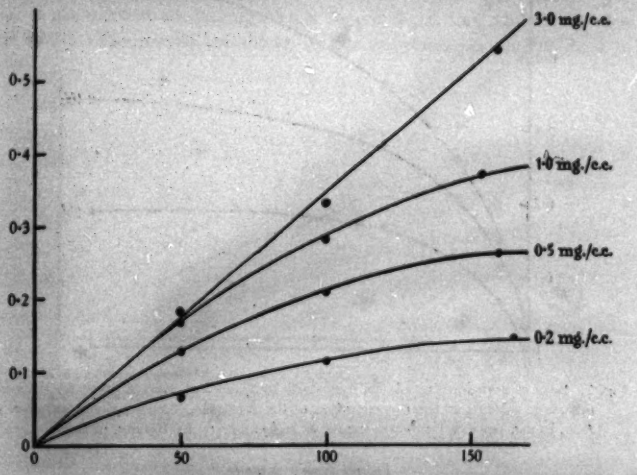

Fig. 1. Time course of acetylcholine (ACh.) hydrolysis by finely cut frog muscle (*Rana esculenta*) for different concentrations. *p*H = 7·2. Temp. 18°. Abscissa: time in min. Ordinate: mg. ACh. hydrolysed by 100 mg. of muscle.

The results of these experiments are also seen in Fig. 1 in which the time course of the hydrolysis for different concentrations of acetylcholine is shown. With two exceptions each point represents the mean of two single determinations. It can be seen that only the initial concentration of 3 mg. per c.c. gave an uniform rate of hydrolysis for the period studied. With lower initial concentrations the rates slowed down during the observations. Since the initial rates of the two highest concentrations—1 and 3 mg. per c.c. respectively—were practically identical, it follows that at this initial concentration saturation was reached.

This is more clearly shown in Fig. 2 in which the initial concentrations of acetylcholine are plotted against the amounts of substrate hydrolysed by 100 mg. of muscle. The points giving the hydrolysis after equal times are connected by curves. It can be seen, e.g. that the amounts hydrolysed in 50 min. are approximately the same for initial concentrations of 1 and 3 mg. per c.c. respectively.

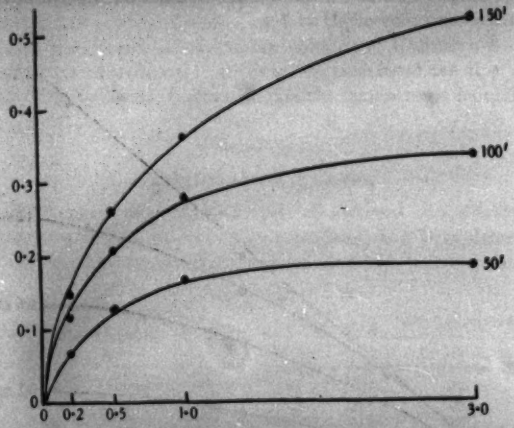

Fig. 2. Rate of acetylcholine (ACh.) hydrolysis by finely cut muscle tissue for different periods in relation to the concentration of the substrate. Abscissa: concentration of ACh. in mg. per c.c. Ordinate: mg. of ACh. hydrolysed by 100 mg. of tissue.

Intact frog's muscle

In experiments in which the effect of acetylcholine on the metabolism of muscle was studied it was found that this effect persisted for many hours [see Marnay & Nachmansohn, 1936]. This suggested that the rate of acetylcholine hydrolysis in intact muscle was slower than could be expected from data so far available in the literature, and it was therefore of interest to determine the rate of decomposition of acetylcholine in the intact muscle with the method described. Since it was desired to follow the time course of hydrolysis in a single experiment and to obtain several points on the hydrolysis curve with tissue from one animal, the muscles chosen for these experiments were the gracilis and semitendinosus of the two hindlegs. These muscles had been found in previous experiments on metabolism to be very similar in weight and properties.

TABLE II

Weight of muscle mg.	Period of hydrolysis min.	Initial amount of ACh. μg.	ACh. remaining μg.	ACh. hydrolysed (μg.) Whole amount	By 100 mg. of muscle
420	55	1087	905	182	43
410	100	1087	765	322	78
410	210	1087	575	512	125

In the experiments shown in Table II each muscle was incubated in 5 c.c. bicarbonate Ringer solution containing 0·2 mg. acetylcholine per c.c. A gas mixture containing 95 p.c. N_2 and 5 p.c. CO_2 again ensured a *p*H of 7·2. The results are shown by the unbroken line in Fig. 3. For

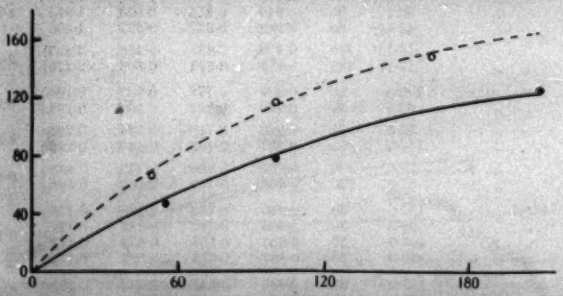

Fig. 3. Time-course of acetylcholine (ACh.) hydrolysis by intact muscle of *Rana esculenta*. Weight of muscle 420 and 410 mg. respectively. Concentration of ACh. 0·2 mg. per c.c. The broken line gives comparatively the time course of hydrolysis by finely cut muscle for the same initial concentration of ACh. Abscissa: time in min. Ordinate: μg. of ACh. hydrolysed by 100 mg.

comparison, the curve found with the same acetylcholine concentration in finely cut muscle (Fig. 1) is drawn in as a broken line. It is seen that the rate of hydrolysis is only slightly smaller in the intact muscle. This shows that the figures obtained for finely cut muscle can be taken, without great error, as indicating the rate of acetylcholine hydrolysis in intact muscle.

Other organs

The same method has also been applied in a few experiments to the determination of the esterase activity of various guinea-pig organs. The Ringer solution for these tissues contained [see Krebs & Henseleit, 1932]: NaCl 0·154 *M*, 100 parts; KCl 0·154 *M*, 4 parts; $CaCl_2$ 0·11 *M*, 3 parts; $MgSO_4$ 0·154 *M*, 1 part; $NaHCO_3$ 0·154 *M*, 10 parts. The

slices were suspended in 5 c.c. of this solution. These experiments were carried out in Warburg vessels kept in a thermostat at 37°. The concentration of acetylcholine was always 0·2 mg. per c.c. The substance was added after adjustment of the temperature. For the rest the procedure was the same as described above.

TABLE III

No.	Tissue	Weight of tissue mg.	Period of hydrolysis min.	Initial amount of ACh. mg.	ACh. remaining mg.	ACh. hydrolysed (mg.) Whole amount	By 100 mg. of tissue	Mean value
1	Brain cortex	80·3	30	0·837	0·231	0·606	0·755	0·755
2	,,	32·3	60	0·966	0·535	0·431	1·335	1·353
3	,,	39·2	60	0·949	0·414	0·535	1·365	
4	,,	42·45	60	0·949	0·372	0·577	1·360	
5	,,	18·15	120	0·876	0·438	0·438	2·410	2·340
6	,,	22·10	120	0·876	0·373	0·503	2·270	
7	Renal cortex	109·0	30	0·980	0·793	0·183	0·168	0·171
8	,,	62·9	30	0·966	0·857	0·109	0·174	
9	,,	92·5	60	0·980	0·716	0·264	0·285	0·266
10	,,	177·0	60	0·837	0·402	0·435	0·246	
11	,,	78·5	120	0·980	0·628	0·352	0·442	0·419
12	,,	47·6	120	0·966	0·777	0·189	0·396	
13	Liver	130·0	30	0·980	0·752	0·228	0·175	0·155
14	,,	96·9	30	0·966	0·780	0·186	0·192	
15	,,	407·0	30	0·837	0·298	0·539	0·132	
16	,,	440·0	30	0·837	0·313	0·524	0·119	
17	Liver	157·0	60	0·980	0·648	0·332	0·212	0·210
18	,,	120·9	60	0·966	0·715	0·251	0·208	
19	,,	149·0	120	0·980	0·519	0·461	0·310	0·364
20	,,	75·0	120	0·966	0·652	0·314	0·418	
21	Uterus	74·25	30	0·876	0·311	0·565	0·760	
22	,,	17·30	120	0·876	0·472	0·404	2·330	2·085
23	,,	17·75	120	0·876	0·550	0·326	1·840	
24	Pregnant uterus	38·8	62	0·837	0·115	0·722	1·850	
25	,,	13·2	120	0·837	0·439	0·398	3·000	
26	Heart	162·45	60	0·949	0·623	0·326	0·205	0·204
27	,,	132·50	60	0·949	0·678	0·271	0·203	
28	Voluntary muscle	350·0	30	0·861	0·229	0·632	0·180	
29	,,	201·0	60	0·861	0·304	0·557	0·277	
30	,,	117·0	120	0·861	0·193	0·668	0·550	
31	Adrenals	94·2	60	0·915	0·663	0·252	0·270	0·298
32	,,	91·45	60	0·915	0·619	0·296	0·325	

Table III gives a survey of the data obtained with brain cortex, renal cortex, liver, striated muscle (hindleg), muscle of uterus, heart muscle (ventricle) and adrenals. The weights given in the table are fresh weights,

these being derived, with a few exceptions, from the dry weights by multiplying by 5.

Fig. 4 shows the curves of hydrolysis obtained from brain, kidney and liver. The few observations with other tissues indicate that the activity

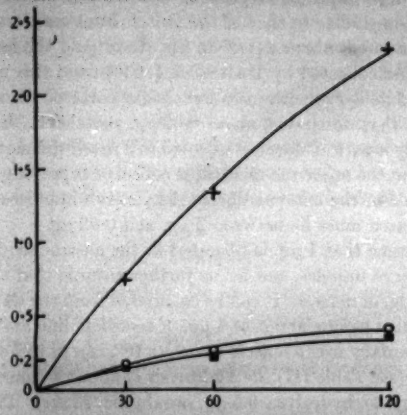

Fig. 4. Time course of acetylcholine (ACh.) hydrolysis by slices of some organs of the guinea-pig. Concentration of ACh. 0·2 mg. per c.c. pH = 7·2. Temp. 37°. + + + brain cortex; ooo renal cortex; ■■■ liver. Abscissa: time in min. Ordinate: mg. ACh. hydrolysed by 100 mg. tissue.

of the uterus is not very different from that of brain tissue and that the data for heart muscle, adrenals and striated muscle lie close to those found for liver and kidney.

Discussion

We have used our data as a basis for calculation of the rate of acetylcholine disappearance under conditions which are of interest in connexion with the chemical transmitter theory of neuromuscular excitation. According to Brown *et al.* [1936] one of the chief difficulties which this theory encounters when applied to the excitation of ganglion cells and voluntary muscle fibres is the brief refractory periods of these quickly reacting cells. The same authors have suggested that a persistent supraliminal concentration of acetylcholine at the end plates will produce a repetitive response of the muscle. The fact that a single motor impulse evokes only a single response of the muscle fibre would accord-

ingly entail, on the chemical theory, a fall in the local concentration of acetylcholine to a subliminal value within the very brief limits of the refractory period. They suggest, therefore, in order to explain so rapid a fall, that the esterase is concentrated at the nerve endings.

Now the dose required to produce in a cat's gastrocnemius a contraction tension similar to that of the twitch from maximal stimulation of the motor nerve is about 2 μg. On the other hand the largest amount of acetylcholine collected by Dale *et al.* [1936] from this muscle during stimulation of its nerve with maximal shocks corresponded only to one-hundredth of that dose—viz. about 0·02 μg. per shock. But as, on the one hand, only a part of the dose injected will reach the motor end plates rapidly, and on the other the quantities collected represent certainly less than the whole of the amount liberated by nerve impulses, the amount actually liberated must lie between 2 μg. and 0·02 μg.

Let us assume that 1 μg. is liberated in the gastrocnemius of the cat by a single nerve impulse, and let us further assume that this liberation occurs in 10 mg. of muscle. It can be calculated from the data above that 5 min. must pass before 50 p.c. of 1 μg. of acetylcholine are destroyed by 10 mg. of voluntary frog's muscle tissue at 18°. As in the warm-blooded muscle at 37° (see Table III) the hydrolysis is about 4 times quicker, the time necessary to hydrolyse 0·5 μg. would be 75 sec. The refractory period of the frog's skeletal muscle is about 5 μsec. and in mammalian muscle even shorter.

The time necessary for hydrolysing 50 p.c. of 1 μg. ACh. in the gastrocnemius of a cat is therefore at least 15,000 times longer than the refractory period of the muscle. The assumption of 1 μg. acetylcholine liberated in 10 mg. is the most favourable. Up to 200 mg. the values are approximately the same, above 200 mg. or below 10 mg. the rate of hydrolysis would be even lower. The esterase activity of nervous tissue (brain cortex) is about six times greater than that of voluntary muscle. The same concentration of enzyme may be admitted in the case of the motor nerves. Furthermore, in the muscle pieces diffusion may be an inhibiting factor which does not intervene at the surface of the nerve endings. In any case, however, a very high concentration of esterase at the end plates is necessary for the hydrolysis, within the limits of the refractory period, of the acetylcholine liberated during the stimulation. It will be of interest therefore to bring some evidence for such a high concentration of esterase at the end plates.

Quastel *et al.* [1936] have recently published a paper in which they determined for several tissues of the guinea-pig the $Q_{CO_2}^{N_2}$, i.e. the anaerobic

liberation of CO_2 by 1 mg. dry weight of tissue in 1 hour, in the presence of acetylcholine. Their procedure differs from that described in the present paper, in that they did not determine the amounts of acetylcholine metabolized directly but assumed that the CO_2 liberation was an adequate measure of the rate of acetylcholine hydrolysis. In some preliminary experiments made before that paper was published, it was observed that the CO_2 liberation of tissue slices after the addition of acetylcholine did not correspond exactly with the disappearance of acetylcholine as found by the method used in this paper. The CO_2 formation was less than was to be expected from the amounts of acetylcholine hydrolysed. Moreover, the authors used concentrations different from those used in our experiments, so that the results obtained are not quite comparable. This point too needs further investigation.

Summary

1. A method is described for determinating small amounts of acetylcholine (0·2–0·6 mg.).

2. The time course of hydrolysis of acetylcholine by finely cut voluntary muscle of the frog (*Rana esculenta*) is determined for different concentrations of substrate.

3. A comparison is made with the rate of hydrolysis by the intact muscle of the frog.

4. The rate of acetylcholine hydrolysis by some tissues of the guinea-pig is determined.

5. The difficulty is discussed of assuming a hydrolysis of acetylcholine within the refractory period of voluntary muscle.

The authors are indebted to Prof. L. Lapicque for the hospitality given to them in his laboratory.

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612.744:592

CHOLINESTERASE IN INVERTEBRATE MUSCLES

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The neuromuscular system in invertebrates, especially in crustaceans, has been the subject of many investigations. The results of Dale and his co-workers, suggesting a humoral mechanism of neuromuscular transmission in higher animals, reopened the question of the nature of the transmission in lower animals. Arguments in favour of a different mechanism of transmission in crustaceans have been discussed recently by Katz [1936] in a paper in which the work of earlier investigators is described.

Since a relation between cholinergic transmission and the presence of acetylcholine or cholinesterase had previously been suggested, Bacq [1935] investigated the distribution of acetylcholine and cholinesterase in invertebrate tissues. He could not find any esterase activity in crustacean muscle extracts, a fact which he interpreted to be in favour of a different kind of neuromuscular transmission in these animals. These experiments were made qualitatively and only by a biological method. It was therefore thought desirable to make quantitative measurements by a method earlier described [Marnay & Nachmansohn, 1937].

Pieces of finely cut muscle were suspended in 5 c.c. of artificial sea water [see Pantin, 1934]: NaCl 0·6 *M*, 1000 parts; KCl 0·6 *M*, 25 parts; $CaCl_2$ 0·4 *M*, 35 parts; $MgCl_2$ 0·4 *M*, 35 parts; $NaHCO_3$ 1·0 *M*, 20 parts. The experiments were carried out in Erlenmeyer flasks, a gas mixture containing 5 p.c. CO_2 and 95 p.c. N_2 was bubbled through the solution during the experiment, thereby regulating the *p*H at 7·5. After a suitable time the slices were removed and an aliquot amount of the fluid taken for the manometric acetylcholine determination. The initial concentration was always 0·2 mg. acetylcholine chloride per c.c.

Table I shows the results obtained. Muscles of five different groups of animals were examined: crustaceans (*Homarus vulgaris*), molluscs (*Eusepia officinalis*), echinoderms (*Holothuria nigra*), annelid worms

TABLE I

No.	Animal	Tissue	Weight mg.	Period of hydrolysis min.	Initial amount of ACh. μg.	ACh. remaining μg.	ACh. hydrolysed μg.	Hydrolysed by 100 mg. of tissue μg.
1	*Holothuria nigra*	Musculus longitudinalis	700	30	1000	742	258	37
2	,,	,,	184	155	1000	643	357	194
3	*Homarus vulgaris*	Musculus abdominalis	410	80	955	521	434	106
4	,,	,,	290	120	955	490	465	160
5	,,	,,	240	160	955	486	469	195
6	,,	,,	140	180	955	640	315	225
7	*Eusepia officinalis*	Muscle	910	60	965	343	622	69
8	,,	,,	390	115	965	371	594	152
9	,,	,,	280	160	965	368	597	213
10	,,	Brain-ganglion	116	21	850	332	518	446
11	,,	,,	43	30	850	650	200	465
12	,,	,,	52	60	850	154	696	1334
13	*Pontobdella muricata*	Skin muscle	333	40	970	884	86	26
14	,,	,,	348	60	970	775	195	56
15	,,	,,	450	120	965	535	430	96
16	*Metridium senile*	Sphincter muscle	2230	120	850	755	95	4

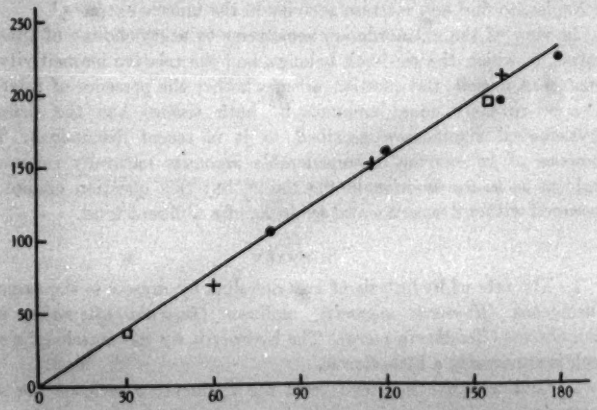

Fig. 1. Time course of acetylcholine (ACh.) hydrolysis by muscle tissue of *Homarus vulgaris*, *Eusepia officinalis* and *Holothuria nigra*. Abscissa: time in min. Ordinate: μg. ACh. hydrolysed by 100 mg. of muscle. + + + *Eusepia officinalis*; □ □ □ *Holothuria nigra*; • • • *Homarus vulgaris*.

(*Pontobdella muricata*, sea-leech) and the sea-anemone (*Metridium senile*). The table also contains the result of an experiment in which the rate of acetylcholine hydrolysis by the brain ganglion of *Eusepia* was determined.

The experiments had the surprising result, that in such different groups as crustaceans, molluscs and echinoderms the rate of the hydrolysis by muscle tissue was exactly the same. This is shown in Fig. 1. The points, which give the results of single determinations, all lie on the same curve. The hydrolysis by the muscle of the sea-leech was apparently a little slower, but this may be due to the fact that the muscle cannot be separated from the skin of the leech. Practically no esterase was found in the sphincter muscle of the sea-anemone: after 120 min. 4 μg. only per 100 mg. of tissue had disappeared. The rate of hydrolysis in the brain ganglion of *Eusepia* was more than ten times greater than that found in the muscle of the same animal. This corresponds with the large amount of acetylcholine found by Bacq in this ganglion. It may be noted that a similar relation was found between the rates of hydrolysis in nervous tissue (brain cortex) [Quastel *et al.* 1936] and the voluntary muscle of the guinea-pig [Marnay & Nachmansohn, 1937].

The results are in agreement with previous qualitative observations by Bacq, except in the case of crustacean muscle. Here he was unable, in Naples, to find any esterase activity in the muscle extracts.[1]

In view of the extraordinary sensitivity to acetylcholine of annelid worms, to which the sea-leech belongs, and the relative insensitivity of crustacean muscle, the question arises whether the presence of esterase in approximately equal amounts in both tissues has the definite physiological significance ascribed to it in recent discussions. The presence of an enzyme in considerable amounts naturally raises the problem as to its function in the tissue, but this question cannot be answered without experimental evidence of a different kind.

Summary

1. The rate of hydrolysis of acetylcholine by muscle is the same in crustaceans (*Homarus vulgaris*), molluscs (*Eusepia officinalis*) and echinoderms (*Holothuria nigra*). The hydrolysis by the muscle of a sea-leech is apparently a little slower.

2. Cholinesterase is absent from the sphincter muscle of the sea-anemone.

3. The hydrolysis of acetylcholine by the brain ganglion of *Eusepia* is more than ten times quicker than by muscle.

[1] This fact will be discussed more fully in another paper. Z. M. B.

The authors are most gratefully indebted to Prof. A. V. Hill for his encouragement; and they wish to thank Dr E. I. Allen and his staff for their kindness and hospitality, and for placing the rich facilities of the Plymouth Laboratory at their disposal.

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OBSERVATIONS IN MAN UPON A BLOOD PRESSURE RAISING REFLEX ARISING FROM THE VOLUNTARY MUSCLES

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This paper is concerned with the rather large changes in the general blood pressure which we observed in man during the muscular exercise of a limb when the circulation of blood through its working muscles was arrested by mechanical pressure. Our earliest observations were made in the course of experiments which were not concerned directly with the present investigation.

Method

Special large sphygmomanometer cuffs were placed round the thighs of a normal subject who was in the sitting posture, and a flat iron weight of 12 kg. was laid across the knees. During the control period the systolic and diastolic blood pressures were measured in an arm by the auscultatory method at about half-minute intervals, until constant readings were obtained. The sphygmomanometer cuffs round the thighs were then inflated rapidly to a pressure well above the subject's systolic blood pressure in order to arrest the circulation through both the legs and further blood pressure readings were taken in the arm. The subject was next instructed to raise and lower the knees. The distance traversed by the weight during each of its excursions was about 5 cm. The movement was performed using as far as possible only the calf muscles. This movement was repeated at intervals varying in the different experiments between 0·5 and 2 sec. The first experiment of a series on any one subject was usually only for the training of the subject and the results then were not recorded.

It was found that both the systolic and the diastolic blood pressures increase as a result of such exercise (Fig. 1). On stopping exercise the blood pressure usually fell only a few millimetres of mercury and remained always above the original normal level until such time as the circulation through the legs was restored by releasing the pressure in the sphygmomanometer cuffs round the thighs. If the circulation remained obstructed for more than 3 or 4 min. after the exercise ceases it was found that a further gradual rise of blood pressure often took place. In the early experiments on the legs there was no exact control of the amount or

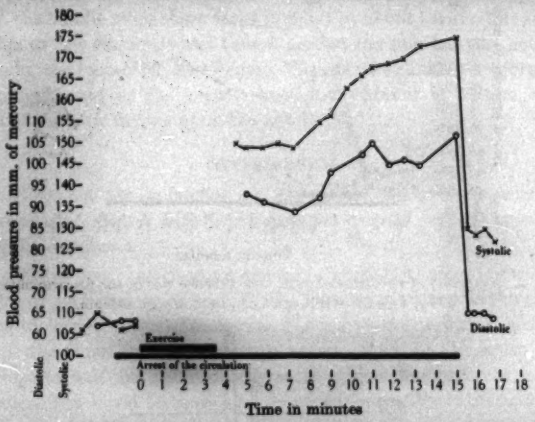

Fig. 1. The exercise was the lifting of 12 kg. through 5 cm. 120 times in 3·5 min. The calf muscles of both legs were the chief muscles involved.

rate of exercise. The degree of increase in the blood pressure which it was possible to secure by this method varied much from one subject to another but was often in the region of 25 mm. of mercury. Increases of as much as 68 mm. of mercury have been encountered.

Experiments were next performed in which the circulation through one forearm was arrested by means of a sphygmomanometer cuff placed round the upper arm and inflated to well above the systolic blood pressure. Exercise, which consisted in squeezing the rubber ball from the sphygmomanometer at intervals of 1 sec. for a few minutes, was performed with the circulation arrested. Increases in the systolic blood pressure of as much as 82 mm. of mercury were recorded. More experiments were made

upon the forearm than upon the leg. It was found that the rise of the blood pressure was established during the exercise and fell very slightly

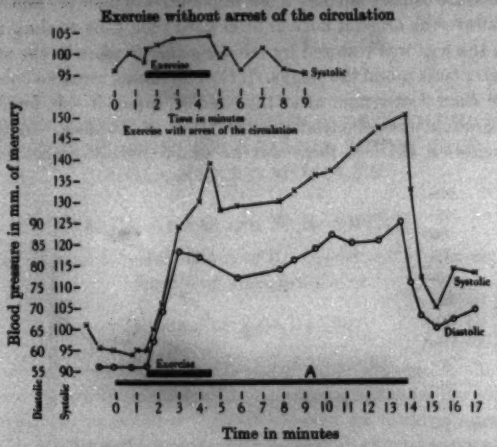

Fig. 2. *A* = period of circulatory arrest. The exercise was of the forearm muscles. 500 g. were lifted through 5 cm. 180 times in 180 min.

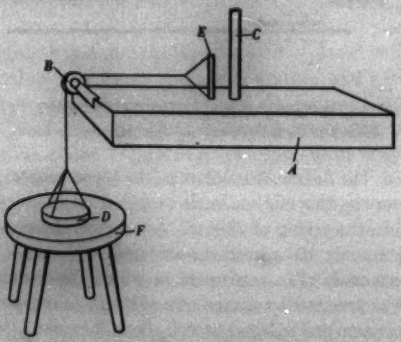

Fig. 3. Apparatus used for exercising the forearm muscles.

on cessation of exercise, but that most of the increase in blood pressure persisted until the circulation through the arm was re-established (Fig. 2).

The experiments which follow were designed to explain the rise of the blood pressure described above. For this purpose it was found necessary to measure the work done by the contracting muscles. Since the forearm is better adapted than the leg for the accurate performance of a muscular task most experiments have been performed with exercise of the forearm muscles. For this purpose the following apparatus was used. A piece of wood *A* (Fig. 3) about 70 cm. long carries a pulley *B* at one extremity and a wide rounded iron bar *C* rises vertically from its centre. A scale pan *D* carrying the desired weight hangs over the pulley by means of a rope which is attached to the handle *E*. When the scale pan rests upon the stool *F* the handle stops short of the iron bar by about 5·5 cm. The subject rests his thenar eminence and thumb against the iron bar and holds the handle by the remaining four fingers. The exercise consists in alternately raising and lowering the weight through a distance of 5·5 cm. by the action of the four fingers upon the handle *E*.

Experimental

(1) *Evidence that the maintenance of the rise in general blood pressure on exercise of muscles with their circulation arrested depends upon arrest of the circulation*

A series of experiments was arranged in which the amount of work and the rate of performance of work by the forearm muscles was the same in each of the experiments of the series. The individual experiments differed only in the duration of circulatory arrest. Each of the series of the experiments was completed on the same subject but different subjects were used. The blood pressure was measured frequently in the arm which was unoccupied by the exercise. Certain changes in the blood pressure were observed and it is evident (Figs. 2 and 4) that the presence or absence of arrest of the circulation through the working muscles of the forearm has a considerable influence upon the blood-pressure readings found in the free and resting arm. When the circulation is free the rise of blood pressure is small and the blood pressure falls to normal within a few seconds after cessation of exercise. When the forearm muscles are exercised while their circulation is arrested, the rise of general blood pressure is much greater than that obtained from equal exercise of the same forearm with the circulation free. If the circulation through the forearm is restored at the end of the exercise the blood pressure falls rapidly and is often near to the normal value in about 20 sec. In numerous other experiments obstruction to the circulation was maintained for various periods of time after cessation of the exercise. It was found that

in normal subjects the rise of blood pressure persisted so long as the arrest of the circulation persisted, and upon the re-establishment of the circulation the blood pressure always fell promptly towards the original normal level.

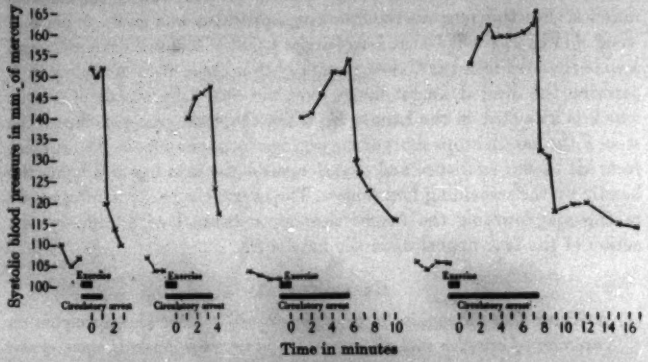

Fig. 4. The exercise was of the forearm muscles. 2 kg. was lifted through 5·5 cm. about 120 times in each experiment.

All of the results of more than fifty other experiments conducted on the same principles as those described above support the conclusion that the maintenance of the rise in blood pressure depends upon arrest of the flow of blood through the working muscle, and that exercise of the muscles of the forearm causes a much greater rise of blood pressure when the flow of blood through the exercising forearm is arrested.

(2) *Evidence that the rise of general blood pressure does not depend on the method of arresting the circulation through the working forearm*

A forearm may be devascularized and the circulation arrested by plunging it into a bath of mercury to a depth of about 12 cm. above the elbow. If the forearm muscles are exercised while the forearm circulation is thus arrested it is found that the systolic and diastolic blood pressures rise and are maintained at a high level until circulation through the arm is restored by withdrawal from the bath of mercury. It is clear therefore that the rise of blood pressure does not depend upon the manner in which the circulation through the forearm is arrested. The temperature of the bath of mercury should be between 33 and 37° C. or rapid cooling of the

forearm while in the mercury may cause changes in blood pressure which are not related to the muscular exercise. A typical result is shown in Fig. 5.

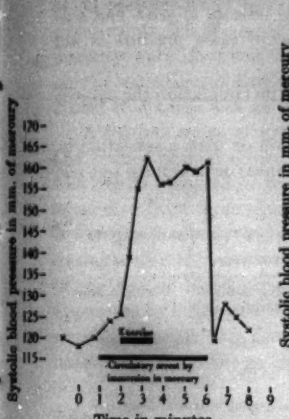

Fig. 5.

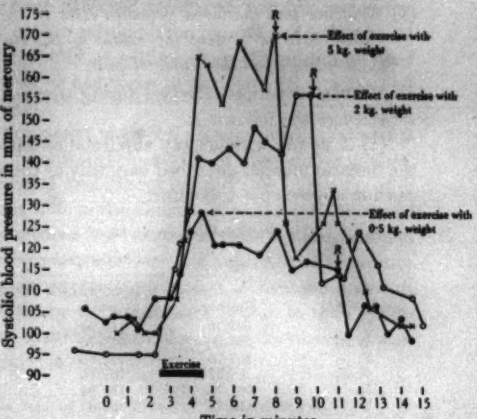

Fig. 6.

Fig. 5. The exercise was of the forearm muscles. A sphygmomanometer bulb was squeezed under mercury about 60 times.

Fig. 6. The exercise was of the forearm muscles during arrest of their circulation. The weights were lifted 60 times. Circulation through the forearm was restored at *R*.

(3) *On exercise of the forearm muscles with their circulation arrested, the extent of the resulting rise of the general blood pressure depends upon the amount of work done by the forearm muscles*

In all the following experiments the circulation through the forearm was arrested during and for some time after exercise. The number of muscular efforts and the distance through which the weight was lifted were constant through any series of experiments performed on the same subject. The weight to be lifted varied from 0·5 to 5 kg. in the different experiments of a series.

Fig. 6 summarizes results of one of the three experiments performed, and it is seen that when our observations are confined to the muscles of the forearm the rise of blood pressure increases with the amount of work done during the circulatory arrest. In over forty experiments it has been shown also that when the work done by the forearm muscles is increased by increasing only the number of muscle efforts then the greater rises of

the blood pressure are still obtained in the experiments where more muscular work is done.

(4) *Evidence that the blood pressure rises in the absence of pain and that when pain is present the extent of the rise of blood pressure is not dependent upon the degree of pain*

When a forearm is exercised during arrest of its circulation the subject experiences the following:

(1) A period without any abnormal sensation or with a sensation in the forearm muscles described variously as tiredness or heaviness but not causing appreciable discomfort.

TABLE I. The relationship between blood pressure and pain in selected subjects

	Blood-pressure measurements			
Subject	Resting	Highest without pain	During slight or moderate pain	During severe pain
G.	108/70	144/94	—	152/100
W.R.	108/60	118/70	130/80	140/88
S.	107/78	120/82	—	—
W.G.	98/60	108/75	114/82	128/88
R.	107/74	147/94	—	—
R.	112/64	150/87	138/89	186/101

Blood pressures below the usual English range are common in Egypt. All readings are made with mercury manometers. Large blood pressure increases, as in Table I, are found only in one-fourth of the normal human subjects studied.

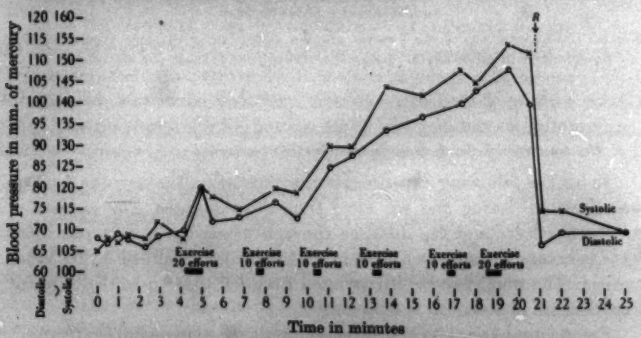

Fig. 7. The exercise was of the forearm muscles. A 2 kg. weight was lifted. The circulation through the forearm was arrested until *R*.

(2) A period where the sensation in the forearm becomes definitely aching in character and is described as pain which, however, is easily tolerated.

(3) A period with severe pain.

It is well known that many types of pain give rise to an increase of blood pressure. Therefore one must consider whether the changes in blood pressure which have been observed are due to pain. In our earlier experiments, made mainly on illiterate subjects, increases of 5–30 mm. of mercury were observed frequently at a time when the subject noticed only a sensation of tiredness or heaviness of the forearm muscles and stated that there was no pain.

A further series of experiments made on doctors, medical students and trained normal subjects confirmed the results of these earlier studies. The results obtained on medical men are set out in Table I (Fig. 7). It is concluded from these results that in the absence of pain a definite rise of blood pressure may be produced by exercise of the forearm muscles during arrest of their blood circulation. In some experiments the course of the rise of blood pressure during the progress of exercise does not appear to be influenced much by the appearance of pain in the forearm muscles (Fig. 7). It is clear that the rise of blood pressure described does not depend upon the production of pain.

(5) *Evidence that the rise of blood pressure is not due mainly to the mental effort involved in exercise*

Experiments have been described in previous sections which indicate clearly that the rise of general blood pressure which develops during the exercise of a limb with its circulation arrested persists after cessation of exercise so long as arrest of the circulation is maintained. It is clear therefore that the mental processes associated with such muscular exercise are not mainly responsible for the rise of blood pressure which takes place.

If the exercise is performed with the circulation normal, the rise of blood pressure is either absent or is much less than in exercise with arrested circulation, yet the mental processes responsible for the muscular contractions are present in both cases.

If the blood-pressure measurements are made at frequent intervals just before and just after exercise ceases, the circulation remaining arrested, it is found almost always that the blood pressure decreases by 5–10 mm. of mercury on cessation of exercise (Fig. 2). This small fall of blood pressure is not progressive and usually is complete within half a minute after the cessation of exercise. It seems likely that the small blood pressure fall is the result of the cessation of mental activity concerned with the muscular exercise.

(6) *Evidence that, with unobstructed circulation, exercise of the forearm muscles, more rapid than previously described in sections* 1–5, *will cause a rise of general blood pressure*

It has been shown already that, when the forearm muscles are exercised slowly with their circulation intact, there is little change in the general blood pressure. If the speed of work is much increased there is good reason to suppose that the flow of blood through the forearm may be insufficient to remove completely the newly formed metabolic products of the exercising muscles. Whatever may be the cause, however, the blood pressure rises appreciably during a rapid exercise of the forearm muscles such as the squeezing of the rubber ball of a sphygmomanometer as frequently as possible.

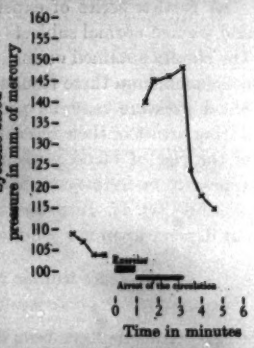

Fig. 8. The exercise was of the forearm muscles. A sphygmomanometer bulb was squeezed 40 times rapidly.

If at the moment exercise ceases the circulation through the forearm is arrested by rapid compression of the upper arm with a sphygmomanometer cuff, it is found that much of the rise of pressure established during exercise persists on cessation of the exercise. As with the experiments described in section 1 the rise of blood pressure is maintained by arrest of the circulation through the arm and falls to normal again whenever the circulation is re-established.

It is clear, therefore, that the part of the rise of blood pressure which persists on cessation of the exercise is not due to mental disturbance resulting from the performance of the exercise. It is clear also that the rise of blood pressure which was demonstrated by slow exercise of an arm with arrested circulation is obtained now by exercising rapidly an arm whose circulation is intact (Fig. 8). The rise of blood pressure thus obtained may be maintained by arresting and maintaining arrest of the circulation after the exercise has ceased.

Discussion

It is well known already that during *general* muscular exercise the blood pressure rises. Several factors may be involved in this blood pressure increase. Thus mental activity associated with the preparation for or the performance of muscular exercise is supposed to cause stimulation of the

vaso-motor centre. The release of adrenaline from the suprarenal glands is thought to play a part in raising the blood pressure during general exercise. The increased venous return to the heart during exercise also is thought to raise blood pressure by increasing the output of the heart. The liberation of acid metabolites of muscle activity into the general circulation is sometimes suggested as an explanation for the rise of blood pressure because of the stimulating action of acid metabolites on the vaso-motor centre. Since a vaso-dilator histamine-like substance is also liberated during muscle activity, the part played by metabolites on their reaching the general circulation must be regarded as doubtful.

Our observations have shown that the activity of even a small group of muscles such as those causing flexion of the little finger of the hand may give rise to an increase of even 70 mm. of mercury in the general blood pressure provided that the circulation of blood through the exercising muscles is arrested completely.

The hypotheses which have been advanced to explain the rise of blood pressure on general exercise do not explain the rise of blood pressure in our experiments. Thus the influence upon the blood pressure of the mental activity associated with muscular exercise is not a very important factor in our experiments because, after cessation of muscular activity, the blood pressure remained at a high level so long as the circulation through the arm was arrested. The mental activity associated with exercise, however, is not quite without influence upon the blood pressure. Some conception of the magnitude of the effect of such mental activity on the blood pressure may be gained from the experiments described in section 5. Increased venous return to the heart due to the greater flow of the blood through active muscles cannot explain the rise of the blood pressure obtained in our experiments since the circulation through the active muscle was arrested. For this reason also the rise of the blood pressure cannot be explained by the release of muscle metabolites into the circulation, for during the period while the blood pressure was high they were confined within the exercised muscles. The effect on the general arterial pressure of sudden arrest of the blood flow through both legs and one arm was found to be negligible in normal subjects. The obstruction was effected very near to the trunk with the object of excluding from the general circulation as much of the limbs as possible. It is clear therefore that the exclusion of a limb from the general circulation will not explain the rise of blood pressure under discussion.

The secretion of adrenaline will not explain the rise of the blood pressure which we have observed for the blood pressure falls at once when

the circulation through the limb is re-established. Moreover, the subjective sensations associated with a rise of blood pressure due to injection of adrenaline are absent from our subjects.

In these experiments the sustained rise of blood pressure which persists after exercise has ceased depends upon the previous performance of muscular exercise at a time when the supply of blood to the working muscles is arrested or when the rate of performance of the work is so great that the supply of blood to the muscles is inadequate. It seems that the maintenance of the rise of blood pressure is due to the retention in the muscle of the products of muscle activity. The rise of arterial pressure takes place when, owing to circulatory arrest, nerves form the only channel of communication between the exercising muscle and the rest of the body. It becomes evident, therefore, that the rise in blood pressure is caused by the passage of nerve impulses out from the exercised muscle. Thus the increase of blood pressure which we have observed on exercise of muscles during arrest of their circulation is due to a reflex. The stimulus which starts and maintains this reflex is the accumulation of muscle metabolites in muscles. Accumulation of metabolites has occurred also in our experiments where the rate of performance of muscular work was rapid. The reflex may be excited strongly when only a small bulk of muscle is involved, thus in one experiment a sustained rise of 70 mm. of mercury followed exercise, with arrest of the blood supply, of the muscles which flex the little finger.

Although exercise of the forearm muscles with arrested circulation causes muscular pain, it was shown that in the course of the exercise the rise of blood pressure often appeared before the pain. In some experiments it was found that exercise of both the legs with arrested circulation produced more pain and a smaller rise of blood pressure than exercise of one forearm in the same subject.

The experiments of section 2 show that the rise of blood pressure does not depend upon the method employed for arresting the circulation. If the forearm is plunged vertically downwards into a bath of mercury the circulation through the lower part of the forearm is arrested because the pressure of the mercury exceeds the systolic blood pressure. Exercise performed with the forearm muscles causes a rise of blood pressure which persists so long as the forearm remains in the mercury. When the forearm is removed from the mercury and the blood flow is re-established the blood pressure falls rapidly to normal.

The rise of blood pressure, although elicited conveniently under a pathological condition, namely, during arrest of the circulation, neverthe-

less occurs under natural physiological conditions. The rise of blood pressure takes place during rapid exercise of the forearm muscles in the absence of any interference with the natural flow of blood. It is clear that during rapid natural exercise the circulation may be insufficient to prevent the accumulation of metabolites with the development of muscle fatigue. The rise of blood pressure obtained under these physiological conditions appears to be the same as the rise of blood pressure obtained when the exercise is performed during arrest of the circulation. It was shown in section 7 that if the circulation is arrested immediately on cessation of rapid exercise performed by the forearm muscles with unobstructed circulation a large part of the rise of blood pressure persists so long as the circulation remains arrested and falls to normal on re-establishment of the circulation. Thus the accumulation of the metabolites formed during natural exercise of the forearm muscles also causes a reflex rise of the general blood pressure.

Conclusions

1. A blood pressure raising reflex arising from voluntary muscles may be demonstrated in normal human subjects by a simple clinical method. The increases of systolic blood pressure, which have been secured by this reflex, range from 5 to 85 mm. of mercury. The increases of the diastolic pressure are usually less than those of the systolic pressure.

2. The reflex is elicited when the substances liberated during muscle work are caused to accumulate in the working muscle either by arresting the circulation through the muscle during its work, or by performing the work under natural conditions but at a rapid rate so that the flow of blood through the working muscle is insufficient to prevent accumulation of the metabolites.

3. High blood pressures produced by the accumulation of metabolites in voluntary muscle may be maintained at a high level by local arrest of the circulation, thus preventing the removal of the excess of metabolites by the blood stream.

4. It seems that this reflex must be designed so that voluntary muscles, as they become fatigued, may call upon the central nervous system to regulate the supply of blood in their favour. The local vaso-dilator action of the muscle metabolites ensures that the blood vessels supplying the muscle will have the full advantage of the blood pressure increase.

5. The rather large blood pressure increases which occur with exercise of small groups of muscles, and under perfectly natural conditions, indicate that this reflex from the voluntary muscles explains in part the rise of blood pressure which is found during vigorous exercise of the whole body.

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FURTHER OBSERVATIONS ON THE ACTION OF ACETYLCHOLINE, PROSTIGMINE AND RELATED SUBSTANCES ON THE KNEE JERK

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In a previous paper we have shown [Schweitzer & Wright, 1937 *b*] that eserine and related compounds and acetylcholine have marked effects on the knee jerk in cats under chloralose anæsthesia. Eserine usually markedly increases the knee jerk, and produces convulsive movements. The response of the quadriceps (of the opposite limb) to direct motor nerve stimulation at the same rate as that at which the knee jerk is elicited (once in 5 or 10 sec.) is as a rule simultaneously enhanced, but evidence was presented that eserine produces its effects on reflexes mainly through a direct excitatory action on the spinal cord. Prostigmine and Stedman's "meta" compound (methyl carbamic ester of *m*-hydroxy phenyl trimethyl ammonium iodide) on the contrary inhibit the knee jerk. As these drugs in the doses used increased the response of the opposite quadriceps to motor nerve stimulation at the rate mentioned, it was concluded that they depress or abolish the knee jerk through a direct inhibitory action on the central nervous system. Acetylcholine also inhibits the knee jerk, and it was demonstrated that although under certain conditions this drug depresses the response of muscle to motor-nerve stimulation, it also has an inhibitory action on the central nervous system.

Rosenblueth *et al.* [1936] and Bacq & Brown [1937] have pointed out that under certain conditions the anti-cholinesterase drugs may depress the response of muscle to motor-nerve stimulation. The latter workers, using the method of close-range injection into the muscle, showed that with appropriate doses of these drugs, the rate of stimulation of the motor nerve influenced the direction of the response. Thus at low

rates of stimulation (once in 10 sec.) potentiation was produced, but when the rate was 24 per min. or over peripheral depression resulted. Wilson & Wright [1936] found in the decerebrate cat that prostigmine injected intra-arterially (into the central end of the inferior mesenteric artery) in doses of 0·1 mg. always produced potentiation of the response of the gastrocnemius stimulated through its motor nerve at the rate of 60 per min. We have repeated and confirmed this result but have found that larger doses of prostigmine may depress the muscular response to motor-nerve stimulation at the above rate. The discharge from the anterior horn cells which is set up when the knee jerk is elicited consists of a slightly asynchronous volley and is, therefore, not exactly comparable with the absolutely synchronous impulse stream produced by motor-nerve stimulation. It is true that in our experiments we have always elicited the knee jerk at intervals of not less than 5 sec., so that the conditions under which Bacq & Brown [1937] obtained peripheral depression of the muscle response were not present. We felt, however, in view of the complex nature of the peripheral action of the anti-cholinesterase group of drugs on skeletal muscle, that it might be unsafe to rely unduly on the behaviour of the quadriceps in response to motor-nerve stimulation as an indisputable index of the way in which the drugs under examination actually modify the peripheral response of the quadriceps when the knee jerk is elicited. We thought it desirable to supplement our previous observations on the mode of action of prostigmine and the "meta" compound on the knee jerk by more direct evidence. We have also repeated some of our observations on acetylcholine using more stringent precautions. In addition, we have studied the action on the knee jerk of other choline esters (doryl and mecholin) and of two other members of the anti-cholinesterase group.

Methods

Cats were used anæsthetized with chloralose (usually 0·07 mg. per kg. body weight). The right hindlimb was rigidly fixed as previously described by means of drills through the ischial tuberosity and the femur. The leg was connected with thread over pulleys to a Sherrington torsion lever myograph. The knee jerk was elicited in the usual way by means of an automatically operated hammer [Schweitzer & Wright, 1937 *a*]. The experiments were usually performed under artificial respiration and after previous administration of atropine in doses of 1 mg. per kg. body weight to annul the depressor action of choline esters and of the anti-cholinesterases on the circulation.

To determine the extent to which drugs under examination act centrally on the nervous system or peripherally on the muscle, it is necessary to cut off the blood supply to the hindlimbs from that to the central nervous system. We have improved on the technique described in our previous paper [Schweitzer & Wright, 1937 *b*] in the following way. The abdominal aorta is exposed and all its branches from the level of the renal arteries or the cœliac axis downwards are carefully isolated and tied with the exception of the right common iliac artery supplying the right hindlimb, the responses of which are recorded. In some instances all branches of the aorta below the diaphragm and also the internal mammaries were ligated. A control experiment is then carried out in which the aorta and inferior vena cava are clamped for periods of 5 to 10 min. or longer while the knee jerk is recorded. It is practically certain that the procedure described prevents any blood from reaching the hindlimb. Under such conditions of complete ischæmia, the force of contraction of the knee jerk may remain unchanged or not uncommonly is initially enhanced[1] (Figs. 1 A, 6 A, 7 A). If the ischæmia is unduly prolonged progressive depression in the knee jerk may be observed. The circulation is then released and the preparation is allowed to recover. Ischæmia is then produced once more and the drug under examination is injected intravenously (into the jugular vein) so as to reach the central nervous system but not the hindlimb.

In some instances the hindlimb was perfused with heparinized blood from another cat by suitably connecting the aortæ and inferior venæ cavæ of the two animals. The injection of drugs into the recipient or donor cat enabled the central or peripheral effects of the substances to be observed.

Finally intra-arterial injections were made into the central end of the inferior mesenteric or renal artery, so as to reach the hindlimb, and the effects were compared with those of intravenous injection of the same dose of the drug.

It was sometimes noticed that during the manipulation of the abdominal contents incidental on clamping the aorta and inferior vena cava temporary depression or abolition of the knee jerk took place (Fig. 7 C). This phenomenon occurred very much more commonly after administration of members of the anti-cholinesterase group. Under these conditions even gentle stroking of the peritoneal surface of the small intestine might abolish the knee jerk and not infrequently the effect persisted for some little time after stimulation had been discontinued.

[1] Dr Brown informs us that he has sometimes observed an increase in the response of muscle to stimulation of its motor nerve during a period of ischæmia.

Results

Action of acetylcholine on the knee jerk

Using animals in which the aorta had been prepared by ligature of all its branches below the renals with the exception of the right common

Fig. 1. Cat. Chloralose (0·085 g. per kg.). Atropine 1 mg. per kg. Artificial respiration. In this and all succeeding figures (except Figs. 5, 8 and 9) the records from above downwards are: right knee jerk (elicited once in 5 sec.), blood pressure, signal line, time in 30 sec. In all experiments in which intra-arterial injections are given or the aorta is clamped it has been previously prepared as described in the text. Intravenous injections are all into jugular vein. A. At "On" clamp aorta and inferior vena cava. At "Off" remove clamps. B. At "On" clamp aorta and inferior vena cava, then inject 2·5 mg. acetylcholine into jugular vein. At "Off" remove clamp. C. Inject 1 mg. eserine intravenously (hindlimb circulation open). At "On" clamp aorta and inferior vena cava; then inject 2·5 mg. acetylcholine intravenously. At "Off" remove clamps. Note secondary decline in knee jerk. After an interval full recovery occurred (D).

iliac, we were able to confirm our previous conclusion that acetylcholine inhibits the knee jerk in part as the result of a central action. Fig. 1 illustrates a representative experiment in an atropinized animal under artificial respiration. During the control period of ischæmia the knee jerk

slightly increased in amplitude (Fig. 1 A). When acetylcholine (2·5 mg.) was injected into the jugular vein during the next period of ischæmia (Fig. 1 B) there was a slight depression of the knee jerk. 1 mg. eserine was then injected and gave rise to an initial depression followed by the customary increase in the knee jerk. 2·5 mg. acetylcholine were injected into the jugular vein once more during a period of ischæmia (Fig. 1 C), and being protected by the anti-cholinesterase action of eserine it produced a sharp marked inhibition of the knee jerk. Recovery to a considerable extent soon took place, although the ischæmia was still maintained. The inhibitory effect must be due to a central action of the drug. On releasing the circulation and so enabling the acetylcholine to reach the hindlimb, there was a considerable secondary decline in the knee jerk, which is to be attributed to a peripheral depressant action of the drug. Gradually full recovery of the knee jerk took place.

Fig. 2. Cat, 3·6 kg. Chloralose (0·065 g. per kg.). 4 mg. atropine. Artificial respiration. At 1, intra-arterial injection into central end of inferior mesenteric 1 c.c. saline. Then increasing doses acetylcholine in 1 c.c. saline as follows: 2, 0·01; 3, 0·05; 4, 0·1; 5, 0·2; 6, 0·5; 7, 1·0 mg. At 8 clamp aorta and inferior vena cava and inject 1·0 mg. acetylcholine into jugular vein. At 9 remove clamps. At 10 inject 1·0 mg. acetylcholine into jugular vein (hindlimb circulation open).

Peripheral action of acetylcholine on the knee jerk

This was further studied in atropinized animals (in which the aorta had been prepared as described) by carrying out intra-arterial injections into the central end of the renal or inferior mesenteric artery. Doses of

acetylcholine under 0·2 mg. are usually without effect. When 0·2–0·5 mg. or more is injected, a transient contracture of the muscles of the hind-limb is produced; with larger doses the contracture is increased in extent and duration (Fig. 2). The knee jerk is simultaneously depressed to a degree which is closely proportional to the extent of the contracture. This depression is most probably the direct result of the contracture [cf. Gasser, 1930]. When the contracture passes off the knee jerk rapidly recovers and not uncommonly shows a secondary rise above its initial level, sometimes to a marked extent. The changes just described are due to a peripheral action of the drug as they are not produced when the same dose of acetylcholine is given intravenously with the circulation to the hindlimb simultaneously blocked. Nor are they produced as a rule following intravenous injection of the same dose with the circulation to the hindlimb open, as under these circumstances the drug reaches the periphery in much lower concentration than after intra-arterial injection. The contracture and associated depression or abolition of the knee jerk are potentiated by previous administration of eserine.

Action of doryl and mecholin on the knee jerk

Doryl (Merck) is the carbaminoyl ester of choline [Kreitmair, 1932]. It has a central inhibitory action which seems to be more powerful and more prolonged than that of acetylcholine. Fig. 3 B illustrates an experiment in which the injection of 1 mg. doryl into the jugular vein during a period of ischæmia of the hindlimb almost completely abolished the knee jerk by a central action. On reopening the circulation to the hind-limb the knee jerk completely disappeared, and recovery did not occur even after 30 min., and in spite of repeated injections of eserine. This prolonged secondary depression is probably due in part to a peripheral action of the drug. In the control experiment (Fig. 3 A) the successive injection of 2 and 4 mg. acetyl choline into the jugular vein during a period of ischæmia of the hindlimb had no effect on the knee jerk. In a few experiments the gastrocnemius was stimulated by means of break shocks to its motor nerve at the rate of once in 5 sec. and various amounts of doryl were injected into the central end of the renal artery after previously preparing the abdominal aorta in our usual manner. The minimum amount of doryl which depressed the response of the muscle was 0·25 mg.; 0·5–1·0 mg. approximately halved the response but even 2·0 mg. did not abolish it completely.

Mecholin (Merck) is the acetyl β-methyl ester of choline [Hunt, 1934; Hunt & Renshaw, 1934]. In our hands the intravenous injection

of mecholin in doses up to 4 mg. in non-atropinized or atropinized animals, with or without previous administration of eserine, had no effect on the knee jerk, although a profound fall of blood pressure, marked slowing of the heart and sometimes œdema of the lung were produced.

Fig. 3. Cat, 3·15 kg. Chloralose (0·085 g. per kg.). 3·5 mg. atropine. Artificial respiration. A. At "On" clamp aorta and inferior vena cava. Then inject 2·0, 4·0 mg. acetylcholine intravenously. At "Off" remove clamps. B. At "On" clamp aorta and inferior vena cava. Then inject 1·0 mg. doryl intravenously. At "Off" remove clamps. After 25 min. interval inject intravenously 2·0, 2·0 mg. eserine.

Action of prostigmine on the knee jerk

(1) *Peripheral action.* Intravenous injection of prostigmine in doses of about 0·25 mg. in non-atropinized or atropinized animals commonly causes a marked increase in the knee jerk, sometimes doubling it. The effect wears off quite slowly, after 10–20 min. It can be readily shown that this increase in the response is due to a peripheral potentiating action of the drug, because if the circulation to the hindlimb is occluded, injection of the same dose into the jugular vein is without effect on the knee jerk.

Similarly, intra-arterial injection of about 0·1–0·2 mg. prostigmine into the central end of the inferior mesenteric or renal artery in animals with the abdominal aorta prepared as described may markedly potentiate the knee jerk response (Fig. 4). When larger doses of prostigmine are

injected intra-arterially (0·2 mg. or over) the knee jerk may be depressed or (with 0·5–1 mg.) abolished temporarily (Fig. 4), and some contracture may also occur. Examined by our usual methods the inhibitory action of prostigmine injected in this way can be shown to be peripheral in nature.

Fig. 4. Cat, 3·7 kg. Chloralose (0·065 g. per kg.). 4 mg. atropine. Artificial respiration. Intra-arterial injections into central end of inferior mesenteric artery successively as follows: 0·1, 0·4, 1·0 mg. prostigmine; then 2 c.c. 3 p.c. KCl on three successive occasions. Note transient contracture in response to first injection of KCl (4th arrow) and depression of knee jerk following second and third injections of KCl (5th and 6th arrows).

Usually the depression of the knee jerk is associated (initially at any rate) with severe "twitching" of peripheral origin. These movements are powerful but writhing and inco-ordinated in character, and so are in-

adequately recorded by the methods we employed to follow extension or flexion of the knee. It is probable that the "twitching" mechanically

Fig. 5. Cat, 4·8 kg. Chloralose (0·065 g. per kg.). 5 mg. atropine. Artificial respiration. Records from above downwards are: contraction of right gastrocnemius stimulated through the sciatic nerve with break shocks at the rate of once in 5 sec., blood pressure, signal line, time in 30 sec. All branches of abdominal aorta below renals, except right common iliac ligated. A. Inject successively 0·25, 0·5 mg. prostigmine into central end of renal artery. B. Inject at 1st arrow intra-arterially 0·5 mg. prostigmine. Inject at 2nd, 3rd and 4th arrows 2·0, 2·0, 2·0 c.c. 3 p.c. KCl. No hastening of recovery of responsiveness is produced. Note, following the second injection of KCl (3rd arrow), there is a transient loss of responsiveness.

hampers elicitation of the knee jerk. This factor does not, however, completely account for the loss of muscle responsiveness, because we have

frequently seen that the knee jerk may remain in abeyance even after the "twitching" has almost completely passed off.

Wilson & Wright [1936] showed that intra-arterial injections of potassium salts have a marked anti-curari action. We have found, however, that injection of potassium salts does not annul the peripheral paralysis produced by prostigmine in skeletal muscle, nor does it hasten the recovery of responsiveness to indirect stimulation. If the muscle has been completely paralysed by means of prostigmine, intra-arterial injection of 2 c.c. 3 p.c. KCl usually produces a transient twitch-like contracture (Fig. 4). This response is in no way dependent on patellar stimulation as it is produced equally well if patellar stimulation is temporarily discontinued during the period of injection of the KCl solution. The contracture is thus the direct result of the potassium acting on the muscle. When the peripheral effect of prostigmine has partially worn off further injections of potassium may be followed by a short period of disappearance of the knee jerk (Fig. 4).

We have examined these points further in experiments in which the gastrocnemius or quadriceps was stimulated by means of break shocks to the motor nerve at the same rate as the knee jerk was elicited in the above experiments, namely once in 5 sec. Intra-arterial injection of prostigmine in small doses (0·1 mg.) potentiates the response, but in larger doses (e.g. 0·5 mg.) it may diminish or abolish the response of the muscle to indirect stimulation (Fig. 5). There is an outburst of "twitching" of marked severity, but when the muscle ceases to respond altogether to nerve stimulation, "twitching" also usually stops; the muscles in other parts of the body, however, continue to twitch violently for many minutes. It must be remembered, however, that the concentration of prostigmine reaching the hindlimb is much greater, because of the intra-arterial route of injection, than in other regions. During the period of paralysis or the onset of recovery repeated intra-arterial injections were made of 2 c.c. 3 p.c. KCl (which has a marked anti-curari action). No beneficial effect on restoration of responsiveness was obtained, and a slight contracture or a few "twitching" movements were the only results noted. If the potassium injection was made when some recovery of the muscle had taken place, it was often followed by a temporary abolition of responsiveness for 20 or 30 sec., as was found in the case of the knee jerk.

The peripheral depressant action of prostigmine on muscle is often described as "curariform". Our experiments suggest, however, that there may be some fundamental difference in the mode of action of

prostigmine and curari. The "paralysis" produced by curari is strikingly annulled by potassium [Wilson & Wright, 1936], but this is not the case with prostigmine "paralysis".

We have made a few further experiments on potassium "contracture". Intra-arterial injection of 2 c.c. 3 p.c. KCl produces a marked "contracture" of the unstimulated quadriceps. Relaxation is usually slow and tremulous in character. In prostigminized muscle the potassium "contracture" is obtained less regularly and the tension developed may be

Fig. 6. Cat, 5 kg. Chloralose (0·07 g. per kg.). 3 mg. atropine. Artificial respiration. A. At "On" clamp aorta and inferior vena cava. At "Off" release clamp. Note increase in knee jerk during period of ischæmia. B. At "On" clamp aorta and inferior vena cava. Then inject successively 1·0, 1·0, 1·0 mg. prostigmine intravenously. Violent "twitching" in forepart of animal but none in right hindlimb. At "Off" release clamps. Violent "twitching" sets in in right hindlimb.

much smaller. Wilson & Wright [1936] found that potassium "contracture" could be very readily produced in chronic motor denervated muscle and passed off more slowly.

(2) *Central action.* To test our previous conclusions on the central inhibitory action of prostigmine, a series of more direct experiments was carried out. The circulation to the right hindlimb was occluded as described and varying amounts of prostigmine were injected into the jugular vein. Doses of 1·5–3 mg. usually produce a diminution or abolition of the knee jerk (Figs. 6, 7). The criterion that was employed to determine whether the occlusion was effective in completely preventing any blood

from reaching the hindlimb was to study the distribution of the "twitching". This occurred regularly with great violence in the forepart of the body, but in satisfactory experiments none whatever took place in the

Fig. 7. Cat, 3·2 kg. Chloralose. Atropine. Artificial respiration. A. At "On" clamp aorta and inferior vena cava. At "Off" release clamps. Note increase in knee jerk during ischæmia. B. At "On" clamp aorta and inferior vena cava and inject 2 mg. prostigmine into jugular vein. Rapid decline in knee jerk; no twitching in right hindlimb. At "Off" remove clamps. Knee jerk disappears at once and severe "twitching" in right hindlimb. Knee jerk recovers gradually. C. At "On" clamp aorta and inferior vena cava. Note complete temporary abolition of knee jerk during abdominal manipulations associated with applying clamps. When knee jerk has fully recovered inject 2 mg. prostigmine into jugular vein. Knee jerk abolished. No "twitching" in right hindlimb. At "Off" remove clamps and "twitching" sets in.

experimental hindlimb. If any trace of "twitching" was noticed in the hindlimb which was being studied, the experiment was regarded as invalid, as it was presumed that some blood containing the drug must have reached the leg through collateral channels, although it is ex-

ceedingly doubtful whether the minute amount in question could have interfered peripherally with the response.

The observations prove that prostigmine depresses reflex activity by an inhibitory action on the central nervous system. This occurs independently of changes in blood pressure, in animals under artificial respiration, and after the injection of atropine.

In some experiments in which prostigmine diminished but did not abolish the knee jerk by a central action during the period of ischæmia, it was found on reopening the circulation to the hindlimb that the knee jerk disappeared coincidentally with the onset of violent "twitching". As has already been suggested these irregular spontaneous contractions of the quadriceps probably interfere mechanically with the extensor movement and so contribute to the failure of the response. In some cases, however, the knee jerk disappeared on reopening the circulation although only mild "twitching" took place (Fig. 7 B). These observations suggest that intensification of the inhibition of the knee jerk which occurs on opening the circulation was due partly to a direct peripheral depressant action of the drug.

Fig. 8. Two cats. Chloralose (0·07 g. per kg.). Artificial respiration. Donor (A) 4·6 kg. and recipient (B) 3·3 kg. heparinized; aortæ and inferior venæ cavæ connected so that hindlimbs of B perfused with blood from A. Records from above downwards are knee jerk (B), blood pressure (B), blood pressure (A), signal line, time in 30 sec. Inject into jugular vein successively 2 mg. prostigmine into A; 1 mg. prostigmine, 1 mg. eserine, 2·5 mg. prostigmine into B.

Cross-circulation experiments point to the same conclusion. Fig. 8 is from an experiment in which the right hindlimb of the recipient (B) was perfused with blood from a donor animal (A). Injection of 2 mg. prostigmine into the jugular vein of the donor (A) caused a small decrease in the knee jerk owing to a peripheral action on the limb of (B). 1 mg. injected into the jugular vein of the recipient (B) to reach the central nervous system caused a more marked and more rapid decline in the knee jerk. The injection of 1 mg. eserine into the jugular vein of the recipient (B) overcame this depression and restored the knee jerk to above its original level. A further injection of prostigmine into the jugular vein of the recipient (B) again caused a depression of the knee jerk. This experiment demonstrates the central inhibitory action of prostigmine and

the central excitory action of eserine. The former can be centrally antagonized by the latter. It also shows further that eserine can markedly stimulate the knee jerk even in the presence of some peripheral depression in the responsiveness of the quadriceps muscle.

Action of Stedman's "meta" compound

Experiments performed along similar lines with the ischæmic hind-limb prove that the "meta" compound also has a central depressant action on the knee jerk. Larger doses also produce peripheral depression of the response of the muscle.

Fig. 9. Cat, 4·2 kg. Chloralose (0·07 g. per kg.). No atropine. Records from above downwards are: knee jerk, respiration (with Wright's quantitative recorder [1934]), blood pressure, signal line, time in 30 sec. Inject 5 mg. "1827" intravenously. Interval of 6 min. between A and B, and 10 min. between B and C.

Action of substance "1827" and "1309" on the knee jerk

These two members of the anti-cholinesterase group were kindly supplied by Roche Laboratories. Substance "1827" is the methyl phenyl carbamic ester and substance "1309" the methyl carbamic ester of *m*-hydroxy phenyl trimethyl ammonium methyl sulphate. They correspond respectively to substances "36" and "13" of the series prepared

by Aeschlimann & Reinert [1931], in whose paper further chemical and physiological details will be found.

(1) In unatropinized animals intravenous injection of about 5 mg. of "1827" abolishes the knee jerk in about one minute. The blood pressure falls initially and subsequently shows large slow rhythmic variations. The heart rate is first increased for about 5 min. and is then slowed for about the same length of time. Respiration is unaffected. The knee jerk may not recover for 10 min. or longer.

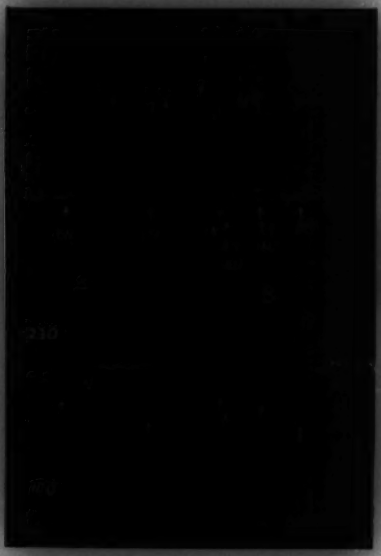

Fig. 10. Cat, 4·0 kg. Chloralose (0·065 g. per kg.). 4 mg. atropine. A. At "On" clamp aorta and inferior vena cava. At "Off" remove clamps. B. At "On" clamp aorta and inferior vena cava. Inject successively 2·5, 2·5 mg. "1827". At "Off" remove clamps. A slight contracture follows while the knee jerk diminishes rapidly. Finally the knee jerk and contracture both disappear.

Essentially the same effects on the knee jerk and respiration are produced in atropinized animals (Fig. 9). The combination of unchanged respiration and absent knee jerk strongly suggests that the drug abolishes the knee jerk predominantly by a central action. This conclusion is only

valid, however, if it is first shown that the peripheral action of "1827" on the diaphragm and other respiratory muscles during breathing and on the quadriceps during the production of the knee jerk is the same. In view of the complex character of the peripheral action of the anticholinesterase group on muscle it would be unsafe to argue in this way in the absence of direct evidence. We have, therefore, further analysed the action of "1827" with our usual technique using the ischæmic hindlimb. Under these conditions the intravenous injection of "1827" still

Fig. 11. Cat, 3·1 kg. Chloralose (0·065 g. per kg.). 4 mg. atropine. Artificial respiration. Between first "On" and "Off", control period of occlusion of abdominal aorta. After 5 min. interval clamp abdominal aorta at "On" and inject into jugular vein 2 mg. "1309". At "Off" remove clamps. Drum subsequently stopped for periods of 35, 10 and 40 min.

depresses the knee jerk by a central action (Fig. 10). We are under the impression, however, from a limited number of experiments, that the decline in the knee jerk sets in more gradually and to a less marked extent under these conditions than when the hindlimb circulation is open. In experiments in which "1827" produced a partial central depression of the knee jerk it was noted that when the blood supply to the hindlimb was restored the knee jerk rapidly declined further (Fig. 10). We have not yet determined to what extent this was due to a continuation of the previous slow central action or to peripheral depression.

(2) Intravenous injection of substance "1309" in unatropinized animals in doses of 1–5 mg. also abolishes the knee jerk, respiration stops soon afterwards and there is marked bronchial spasm. In animals under artificial respiration the drug still produces a fall of blood pressure and slowing of the heart. The knee jerk is not restored by subsequent injection of atropine. This has been our experience with all the depressant anti-cholinesterases so far examined. Left to itself the preparation may recover after a long interval (up to 90 min.).

In atropinized animals intravenous injection of similar doses of "1309" may produce little change in blood pressure, but respiratory arrest may still occur and the knee jerk is readily abolished. Using the ischæmic hindlimb technique, we have found that substance "1309" has a marked central depressant action when injected intravenously in doses of 1–2 mg. (Fig. 11).

Discussion

The results described confirm our previous conclusion that acetylcholine, prostigmine and Stedman's "meta" compound produce an inhibitory action on the central nervous system. It has been shown also that another choline ester (doryl) and two other anti-cholinesterases act in a similar manner. There is no doubt, however, that in the intact animal certain doses of these drugs also exert a peripheral depressant action of varying importance on the response of the muscle. We have found with prostigmine, for example, that when it is allowed to act only on the central nervous system, it may produce its depression of the knee jerk more gradually and less completely, and only when larger doses are employed, than is the case when the circulation to the hindlimbs is open. In this case, therefore, the peripheral depressant action is often of considerable importance. Our evidence with respect to the substances "1827" and "1309" points in the same direction.

Our conclusion about the central inhibitory action of prostigmine has been confirmed by the clinical observations of Kremer, Pearson & Wright [1937]. They have shown that the introduction of 1 mg. of prostigmine into the cerebrospinal fluid by means of lumbar puncture in patients with hemiplegia decreases or abolishes the tendon reflexes and muscle tone in the legs, and sometimes in the arms also, without interfering with sensation. Voluntary movement on the affected side is modified in various ways. In some instances it is depressed, in others it is increased in range and power. The improvement in movement which is sometimes observed is probably due to the abolition of the spasticity

which has been brought about by the drug. In these experiments there can be little doubt that prostigmine produces its inhibitory effects directly on the spinal cord. A similar amount of prostigmine injected subcutaneously or intra-muscularly has no such depressant effect on reflexes. It is very unlikely that the effects of prostigmine are due to local alteration in the circulation in the cord. It is not known to constrict blood vessels by a direct action and, even if it did, we have shown that moderate degrees of anæmia of the cord produce an increase in reflex responses [Schweitzer & Wright, 1937 *a*]. A degree of anæmia sufficient to abolish reflex action would almost certainly depress sensation as well. This, however, does not occur. The fact that sensation is unaffected clinically also suggests that prostigmine acts by depressing directly the discharge of the anterior horn cells rather than by interfering with excitatory transmission through synapses.

It is generally supposed that, when an anti-cholinesterase has the same action in the body or on an isolated tissue as acetylcholine itself, it produces its effects by delaying the destruction of small amounts of naturally liberated acetylcholine. By analogy it may be plausibly argued that the depressant action on the central nervous system of prostigmine and certain other anti-cholinesterases is likewise due to the preservation of naturally formed acetylcholine in the central nervous system. The further conclusion would be that acetylcholine is in some way normally related to physiological processes in the central nervous system. Great caution, however, must be exercised when attempts are made to assign a physiological role to chemical substances on the basis of pharmacological experiments only. The observations recorded in this paper on the peripheral action of prostigmine show how the dose, route of administration and method of stimulation may profoundly influence the type of result obtained.

Dale *et al.* [1936] showed that curari does not prevent the formation of acetylcholine at motor-nerve endings in skeletal muscle but prevents it from exerting its normal physiological action. The peripheral paralyzing action of the anti-cholinesterases is attributed to resulting persistence of acetylcholine, which makes the newly formed acetylcholine less effective as a stimulus [Brown *et al.* 1936]. Our observations with potassium lend further support to the view that the peripheral action of curari differs from the so-called "curariform" action of prostigmine. The "block" induced by curari can be overcome by potassium which is, however, incapable of abolishing prostigmine paralysis. In amplification of the findings of Bacq & Brown [1937] we have shown that with rates

of indirect stimulation as low as once in 5 sec. small doses of prostigmine have a peripheral potentiating and larger doses a paralysing action on muscle.

Conclusions and summary

1. The actions of acetylcholine, doryl and mecholin and several members of the anti-cholinesterase group (prostigmine, Stedman's "meta" compound, substances "1827" and "1309") on the knee jerk were examined in cats under chloralose anæsthesia.

2. All these drugs (except mecholin) depress the knee jerk partly by a central and partly by a peripheral action. The relative importance of these two actions varies with the individual drug and the dose employed.

3. Small doses of prostigmine (0·25 mg. intravenously) increase the knee jerk by a peripheral potentiating action on the response of the muscle to indirect stimulation. Larger doses have a peripheral paralysing action which differs from that of curari in that it is not annulled by appropriate doses of potassium.

The expenses of the above research were partially defrayed by means of a Government grant through the Royal Society.

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ÆTIOPORPHYRIN AND HÆMOGLOBIN REGENERATION AFTER HÆMORRHAGE

By J. HOWELL HUGHES and A. L. LATNER

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(*Received* 17 *February* 1937)

This investigation was carried out in order to find exactly what changes the body could produce in converting a porphyrin into hæmoglobin. Ætioporphyrin was selected on account of the fact that its side chains are pure alkyl derivatives, as seen in Fig. 1. In order to convert the porphyrin into hæmin, the body would have to desaturate two of the ethyl groups and add a carboxyl grouping to the other two. It was considered of some interest to see whether this could be done.

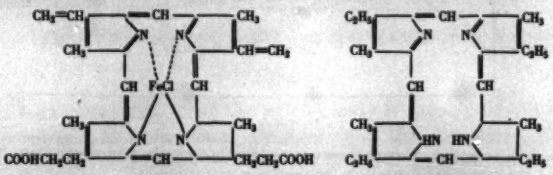

Fig. 1. Chemical structures of hæmin and ætioporphyrin.

The opportunity was also taken to investigate whether there was any difference in the results of administration of large and small doses of the porphyrin. Such a difference has already been shown in the case of chlorophyll in a previous communication [Howell Hughes & Latner, 1936].

Methods

Rabbits have been rendered anæmic by repeated hæmorrhages from an ear vein and then allowed to recover. During their recovery period the test animals were fed with ætioporphyrin dissolved in cod-liver oil, and the controls received equal amounts of this oil. Since the oil had to

be heated in order to dissolve the porphyrin, the oil given to the controls was heated previous to administration.

The procedure used was exactly the same as in the communication to which we have already referred.

Two experiments were performed, one with a relatively large, and the other with a small daily dose of ætioporphyrin. The porphyrin used was kindly supplied to us by Prof. Hans Fischer, and we wish here to record our thanks to him.

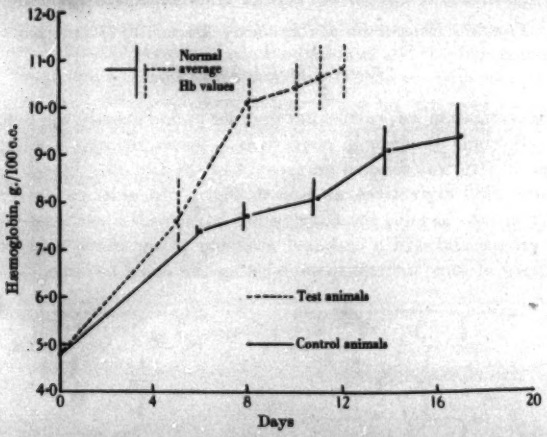

Fig. 2. Administration of larger doses (0·1 g.) of ætioporphyrin. The vertical lines, in this and the subsequent figure, join the maximum and minimum values for each group.

Experimental results

(1) *Larger doses of ætioporphyrin*

0·1 g. ætioporphyrin dissolved in 2 c.c. cod-liver oil was administered daily during the recovery period. Three control animals and four test animals were used, and the average results are shown in graph form in Fig. 2. It is obvious that this dose of ætioporphyrin exerted a favourable effect on blood regeneration.

(2) *Smaller doses of ætioporphyrin*

0·035 g. ætioporphyrin dissolved in 2 c.c. cod-liver oil were administered daily. Three control animals and four test animals were used. The results

are shown in Fig. 3. Although the ætioporphyrin has obviously exerted a favourable effect on hæmoglobin regeneration, the test animals required a longer period to recover than in the previous experiment.

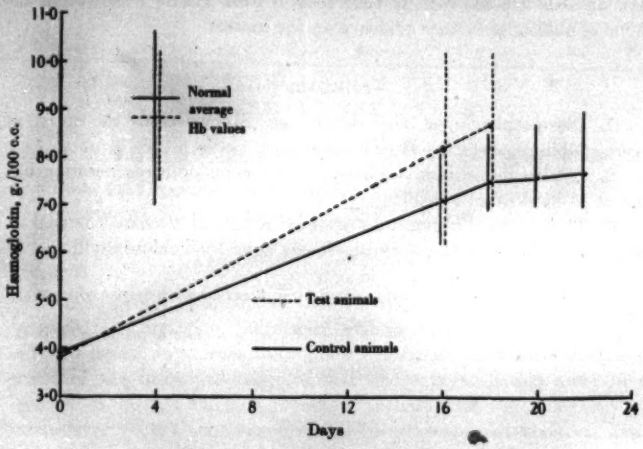

Fig. 3. Administration of smaller doses (0·035 g.) of ætioporphyrin.

Discussion

It seems, therefore, that the animal body is capable of converting ætioporphyrin to hæmoglobin, irrespective of the dose given. This is interesting as proof of the fact that the body can produce the chemical changes already mentioned. It seems reasonable to suggest, therefore, that the body can convert most porphyrins present in the diet into hæmoglobin. It is hoped to reinforce this statement by the results of later experiments.

The fact that the larger doses of ætioporphyrin were more effective than the smaller ones, shows that the toxicity to the bone marrow observed with large doses of chlorophyll in our previous communication is not a general property of the porphyrin group. This is, in a way, further support of the view that it is the magnesium present in the chlorophyll which is a toxic factor, as this element is not an intrinsic part of the porphyrin molecule.

In the case of ætioporphyrin, as with chlorophyll, the part played by intestinal bacteria might possibly be of some significance. Ætioporphyrin

cannot readily be administered subcutaneously owing to the difficulty of getting it into solution; if dissolved in cod-liver oil it is feared that the absorption would be too slow to be of any use. The fæcal material of the test animals has accordingly been sent to Prof. Hans Fischer in the hope of obtaining further evidence on the matter.

Summary

1. Ætioporphyrin in large doses markedly increases the speed of hæmoglobin regeneration after hæmorrhage.

2. Smaller doses increase the speed of hæmoglobin regeneration but not to so marked an extent.

3. The chemical significance of these results is discussed as well as their connexion with the work previously done with chlorophyll.

In conclusion we should like to thank Prof. H. E. Roaf for much helpful criticism and advice.

Part of the expenses of this research were defrayed by a grant to one of us (J. H. H.) from the British Medical Association.

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AFFERENT FIBRES FROM THE STOMACH AND SMALL INTESTINE

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(*Received* 18 *February* 1937)

It is now generally accepted that though many of the viscera are sensitive to stimulation, the stimuli which are effective for such tissues differ in many ways from those known to be effective on the skin. A study of the literature suggests that many investigators have been ignorant of this fact and have therefore concluded that the intestines were insensitive. Clinical and experimental investigations have shown, however, that increased tension is an adequate physical stimulus to these organs.

Mayer & Pribram [1872] and Brodie & Russell [1900] working with animals demonstrated that distension of the stomach caused reflex changes in blood pressure, and more recently Kappis [1913] and Fröhlich & Meyer [1922] have shown that distension of the gut elicited manifestations of pain. Distension of the gall bladder, bile duct and ureter likewise caused reflex vascular changes [Sherrington, 1899], reflex muscular contractions such as the viscero-pannicular reflex [Ashkenaz & Spiegal, 1935] or pain manifestations [Schrager & Ivy, 1928]. Spasm of the intestinal muscle has been shown by Fröhlich & Meyer [1922], and by Brüning & Gohrbandt [1923], among others, to cause pain by increasing the tension exerted on the nerve endings.

Similar observations have been made on man. Hurst [1911] found that at levels below the œsophagus, distension was the only adequate physical stimulus for sensation. Payne & Poulton [1927] later observed that pain was caused in the œsophagus by distension. Many other clinicians have recorded similar results from various parts of the gut [i.e. Bloomfield & Polland, 1931]. As in animals, spasm of the

intestine wall was found to cause sensations of pain [Mackenzie, 1919; Ryle, 1929]. Boyden & Rigler [1934] described experiments in which the mucosa of the stomach and duodenum was stimulated electrically, the gut outline being watched on the screen. Stimuli of sufficient strength caused a sphincteric contraction of the muscle with an immediate sensation of pain.

It is difficult to correlate the results of many of the experiments, as details of the mode and degree of stimulation are not given. A summary of the information available is given in Table I. It will be seen that the pressure used by different investigators to elicit a response in corresponding regions varied considerably.

TABLE I

Author	Organ	Pressure	Sensation
Hurst [1911]	Œsophagus	44 mm. Hg	Fullness
„	Stomach	12 mm. Hg	„
Payne & Poulton [1923]	Œsophagus	28 cm. water	Pain
„ „	Stomach	11 cm. water	„
„ „	Stomach (pyloric end)	15 cm. water	„
„ „	„ „	20 cm. water	Severe pain
„ „	Duodenum	20 cm. water	Pain
„ „	„	30 cm. water	Severe pain
„ [1927]	Œsophagus	20 cm. water	Pain
Schrager & Ivy [1928]	Bile ducts and gall bladder	80–200 mm. Hg	„
Polland & Bloomfield [1931]	Œsophagus	90 mm. Hg	„
Bloomfield & Polland [1931]	Duodenum	60 mm. Hg	Pressure
„ „	„	60–120 mm. Hg	Pain

Hurst [1911], Payne & Poulton [1927], and Bloomfield & Polland [1931], actively distended the viscus with a small balloon, and Payne & Poulton [1923] took records of the pressures caused by contraction of the viscus and noted the levels at which pain occurred. Schrager & Ivy [1928] distended the gall bladder or bile ducts of dogs, but the pressures used were extremely high. More recently, Ashkenaz & Spiegal [1935] have shown that distension of the same areas with high pressures will evoke the viscero-pannicular reflex.

As regards the visceral afferent pathways from the abdomen, all investigators are agreed that the splanchnic nerves carry afferent fibres from the upper parts of the gut. Further, it has been shown that impulses conducted by these afferent fibres may cause sensations of pain which can be abolished by section of the splanchnic nerves or of the sympathetic chain, or by infiltration of the nerve trunks with anæsthetic solutions.

There is not, however, such unanimity of opinion with regard to the existence of afferent fibres in the vagus nerves from the abdomen. Lennander [1906] and Foerster [1927] and others have denied the existence of "pain" fibres in the abdominal vagus, and recently Cannon [1933] has stimulated the intact vagus in conscious animals without causing any discomfort or distress. Heinbecker & O'Leary [1933] stimulated the vagus below the inferior laryngeal branch in unanæsthetized cats, and the animals reacted to the stimulation as they had to painful stimuli.

The work of Harper *et al.* [1935] has shown that afferent fibres present in the cat's abdominal vagus nerves enter (i) the spinal cord by the dorsal roots, and (ii) the medulla with the vagus nerves. Impulses conducted by these fibres travel at least as high as the mid-brain. Foerster considers that the afferent impulses in the abdominal vagus are concerned with sensations such as nausea and also elicit reflexes such as vomiting, a view which is supported by the observations of Neuman [1914], who elicited vomiting in cats during stimulation of the central end of the cut abdominal vagus, though he thought that this procedure also caused pain. Goldberg [1931] also demonstrated the vomiting reflex from a distended pyloric pouch. The afferent side of this reflex was entirely through the vague nerves. Shawe [1922] has reported cases of gastric crises in tabes dorsalis in which appropriate posterior rhizotomy has relieved pain, but has not influenced crises in which nausea and vomiting, and pain in the epigastrium and head were the predominant symptoms. Shawe therefore considers the vagus nerves to be responsible for these latter symptoms. Bloomfield & Polland [1931] noted that nausea, retching and referred pains in the head sometimes occurred on distension of the stomach, and they interpreted these symptoms as being vagal in origin.

It is well recognized that the mesentery is sensitive to changes in tension. Its afferent innervation is stated to be partly somatic and partly visceral, the latter fibres running in the splanchnic nerves. Sheehan [1933] points out, however, that the vagus nerves do not contribute to the afferent innervation of the mesentery.

The technique which has been fully described elsewhere [Bain *et al.* 1935] in which dilatation of the pupil was taken as an index of afferent impulses, appeared to us to offer a delicate method of investigating the problem of visceral sensibility. McDowall [1925], using the same technique, found that stretching the gut caused dilatation of the pupil, but cutting did not have the same effect.

We report in this paper the results of our studies on the sensitivities

of the various parts of the upper alimentary tract and the nerve pathways involved, using dilatation as an index of afferent impulses.

A series of experiments was also performed in which the changes in blood pressure resulting from distension of the viscera were recorded.

METHODS

(a) *Reflex dilatation of the pupil*

As in our previous investigations all our observations have been made on the cat. Anæsthesia was induced with ether and continued with chloralose (Martindale), 0·075–0·09 g. per kg. The abdomen was opened by a median incision, and during the operation the stomach and intestines

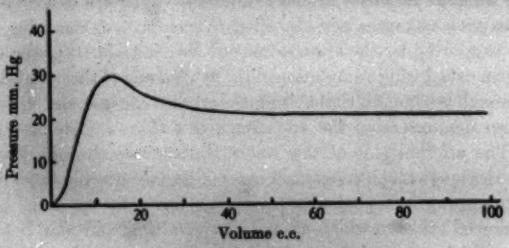

Fig. 1. Calibration curve of typical balloon.

were kept covered with lint soaked in warm saline. Latex rubber balloons of various sizes, attached to narrow rubber tubing, were inserted into the intestine through small incisions running parallel with the mesentery. The balloons were fixed in position by closing the wound in the intestine with a purse-string suture or ligature which held the rubber tubing. To examine the sensitivity of the stomach, balloons were inserted as described later in this paper. After the necessary procedures had been completed, the rubber tubes attached to the balloons were brought to the exterior and the abdomen closed with two layers of sutures. If the cord was divided, the transection was usually carried out after the abdominal operation had been completed. The animal was then left undisturbed for 1–1½ hours. At the end of this time the pupils had usually constricted to a slit, and observations were then made as previously described [Irving & McSwiney, 1935].

The pyloric and intestinal balloons were calibrated, and a typical curve relating pressure and volume is given above. A larger balloon was

inserted into the fundus and body of the stomach. The pressure in mm. Hg and the volume of air in c.c. required to evoke a response were recorded. Using the calibration curve for the balloon it was possible to ascertain approximately the pressure exerted by the wall of the viscus.

(b) Reflex blood-pressure changes

The response of the blood pressure to distension of the stomach and intestine was recorded in the decerebrate and in the spinal cat and in the cat anæsthetized with chloralose.

Experimental results

General

We observed early in our experiments that manipulation of the stomach or intestine after entry into the abdomen caused marked dilatation of the pupil. These reactions were not due solely to stimulation of nerve endings of the abdominal wall or parietal peritoneum, since tension on the duodenal mesentery still caused dilatation of the pupil after section of the spinal cord at the level of the seventh thoracic roots, which rendered the abdominal wall and parietal peritoneum insensitive. In view of these results we considered it advisable to examine, first, the response of the mesenteries to stimulation, and, second, the afferent pathways concerned.

Mesentery

In the first series of experiments the spinal cord was transected below the level of the seventh thoracic roots to obviate any possible interference from the abdominal wall and parietal peritoneum. Very slight tension on the duodenal and jejunal mesenteries sufficed to cause dilatation of the pupil, which persisted after section of the vagus nerves in the neck, but was abolished by section of the splanchnic nerves in the abdomen.

Later, the spinal cord was divided, in some animals between the second and third cervical roots, and in others just below the second thoracic roots. By this procedure afferent impulses carried by fibres in the somatic nerves and in the splanchnic nerves were cut off. Tension on the gastric and duodenal mesenteries still elicited dilatation of the pupil, which, however, was abolished on section of the vagus nerves in the neck. Traction on the jejunal mesentery did not elicit a response when the vagus nerves were the sole conducting pathway.

We therefore conclude that the gastric and duodenal mesenteries receive an afferent innervation from fibres in the vagus and splanchnic

nerves, while the jejunal mesentery is supplied by fibres from the splanchnic nerves.

Stomach and small intestine

Using pupillary dilatation as an index of impulses conducted by afferent nerves in the vagus and sympathetic nerves, we have confirmed the observations of previous investigators that the intestine is insensitive to stimuli which would be painful if applied to cutaneous or deep end organs. Cutting the intestinal wall, burning and scraping the serosa and mucosa failed to elicit a response. Stretching the intestinal wall, however, caused an immediate and marked dilatation of the pupil. Similarly the injection of barium chloride into the wall produced a prolonged muscular spasm and caused immediate pupillary dilatation. We therefore attempted to ascertain (1) the sensitivity and (2) the innervation of the different regions of the digestive tract.

The sensitivity of the stomach and intestines to distension by balloons has been tested. It will be seen that different regions varied markedly in their sensitivity.

Stomach

(*a*) *Fundus and body.*

The fundus and body were distended by a balloon inserted down the œsophagus or through the pyloric sphincter. The antrum was tied off from the body of the stomach by a tape ligature.

The threshold pressure on distension of the body of the stomach varied from 10 to 60 mm. Hg. The response of the pupil appeared to be due to impulses conducted by afferent fibres in the splanchnic and vagus nerves, for after section of the phrenic nerves and transection of the spinal cord at the level of the sixth thoracic roots, dilatation was obtained on distension of the balloon. The response was still obtained after section of the vagus nerves in the neck, but was abolished on subsequent division of the splanchnic nerves. Similarly in other experiments the response was obtained after section of the splanchnic nerves and was abolished by section of the vagus nerves in the neck.

The results of two typical experiments are given in Table II A.

TABLE II A

1.	Vagus and splanchnic nerves intact 25 mm. (100 c.c.)	After section of the vagus nerves in the neck 25 mm. (110 c.c.)	After section of the splanchnic nerves No response
2.	Vagus and splanchnic nerves intact 15 mm. (30 c.c.)	After section of the splanchnic nerves 25 mm. (40 c.c.)	After section of the vagus nerves in the neck No response

(*b*) *Antrum.*

In a further series of experiments the sensitivity of the antrum was examined. A small balloon on a rubber catheter was inserted through an incision in the upper duodenum and passed through the pyloric sphincter into the antrum. The antrum was tied off from the body of the stomach by a loose tape ligature in the region of the incisura angularis. A tight duodenal ligature ensured that the balloon did not slip and stretch the pyloric sphincter. The pressure of the tape ligature between the antrum and the body of the stomach did not interfere with the nerve supply, as the ligature was threaded round the stomach so as not to include the vagus fibres of the lesser curvature.

We found that afferent fibres from the antrum of the stomach were contained in both the vagus and the splanchnic nerves. Results of two typical experiments are given in Table II B.

TABLE II B

1.	Vagus and splanchnic nerves intact	After section of the vagus nerves in the neck	After section of the splanchnic nerves
	20 mm. (4 c.c.)	35 mm. (4 c.c.)	No response
2.	Vagus and splanchnic nerves intact	After section of the splanchnic nerves	After section of the vagus nerves in the neck
	20 mm. (3·5 c.c.)	20 mm. (3 c.c.)	No response

Pyloric sphincter and upper duodenum

To examine this region a small balloon attached to a rubber catheter was inserted through an incision in the first part of the duodenum into the pylorus. The distension of the balloon stretched the pyloric sphincter and the first half to one inch of the duodenum. A loose tape ligature was usually threaded round the proximal part of the antrum to prevent the balloon slipping into the stomach.

In experiments in which balloons had been inserted into both the pyloric sphincter and the duodenum, it was invariably found that the threshold for the pyloric sphincter was lower than that for the duodenum. In some experiments pressure as low as 3 mm. Hg elicited a response from the pyloric sphincter. That the sphincter was innervated by both the vagus and the splanchnic nerves is shown by the following two typical experiments (Table II C).

TABLE II C

1.	Vagus and splanchnic nerves intact	After section of the vagus nerves in the neck	After section of the splanchnic nerves
	10 mm. (1·5 c.c.)	12 mm. (1·5 c.c.)	No response
2.	Vagus and splanchnic nerves intact	After section of the splanchnic nerves	After section of the vagus nerves in the neck
	10 mm. (2 c.c.)	15 mm. (3 c.c.)	No response

Duodenum

In order to test the sensitivity of the duodenum a balloon was inserted into the duodenum below the pyloric sphincter. Care was taken to see that afferent fibres of the vagus nerves running along the intestine should not be included in the ligature which held in position the rubber tube attached to the balloon. Some of the results obtained are given in Table II D. The distance of the tip of the balloon from the pyloric sphincter is given in column one.

TABLE II D

Distance from pyloric sphincter	Vagus and splanchnic nerves intact	After section of the vagus nerves in the neck	After section of the splanchnic nerves
4 cm.	15 mm. (1 c.c.)	18 mm. (1 c.c.)	No response
5 cm.	15 mm.	30 mm.	No response
10 cm.	25 mm. (2 c.c.)	25 mm. (2 c.c.)	No response

Distance from pyloric sphincter	Vagus and splanchnic nerves intact	After section of the splanchnic nerves	After section of the vagus nerves in the neck
3 cm.	15 mm. (2 c.c.)	20 mm. (2 c.c.)	No response
7 cm.	20 mm. (2 c.c.)	40 mm. (2 c.c.)	No response
8 cm.	15 mm. (2 c.c.)	25 mm. (3 c.c.)	No response
9 cm.	30 mm. (3·5 c.c.)	40 mm. (3·5 c.c.)	No response

These results are typical of those obtained, but throughout the series there was considerable variation in the threshold for similar areas of the duodenum in the different experiments.

By dividing the vagus or splanchnic nerves separately during the course of an experiment, we have been able to demonstrate that the stomach and duodenum are innervated by the right and left vagus nerves and by the right and left splanchnic nerves.

Jejunum and ileum

To obtain information of the afferent innervation of the remainder of the small intestine, balloons were inserted into the lumen of the small intestine so as to distend (*a*) the proximal 2 in. of the jejunum, (*b*) 2 in. of the mid-region of the ileum, and (*c*) the ileum just proximal to the ileo-colic sphincter. In a number of experiments the spinal cord was cut in the lower thoracic region. A series of observations was also made with the cord intact to ascertain if afferent fibres entered the cord in the lower thoracic and lumbar regions.

Section of the vagus nerves in the neck did not appear to affect the threshold of the jejunum and ileum, but section of the splanchnic nerves, leaving the vagus nerves as the sole afferent pathway, usually abolished the response. However, in one experiment the threshold remained the same with only the vagus nerves conducting.

The lowest value obtained for the jejunum was 55 mm. Hg and for the mid-ileum 80 mm. Hg. In many experiments pressures greater than 100 mm. Hg were required to elicit a response. Our conclusions, however, are based on observations where the threshold pressures were below 100 mm. Hg. In the lowest part of the ileum a pressure of 200 mm. Hg occasionally caused a very slight response, but in general this area was insensitive to distension by balloons. Section of the cord did not appear to influence the thresholds as similar results were obtained when the spinal cord was left intact.

Observations on blood pressure

Peristalsis was seldom observed in cats anæsthetized with chloralose, and it might be argued that the anæsthetic acted as a general depressant of the visceral end organs, an effect which would account for the insensitivity of the jejunum and ileum. We therefore made a further series of experiments recording alterations of blood pressure in the decerebrate cat, consequent on distension of the intestinal wall as an index of visceral afferent impulses.

A fall in blood pressure was observed on distension of the intestine. The minimum pressure required in different experiments to elicit a response from the pyloric sphincter and upper duodenum varied between 10 and 30 mm. Hg. Reflex vascular changes were recorded after section of both vagus nerves. Section of the splanchnic nerves abolished the response.

Negative results were obtained on distension of the jejunum below 100 mm. Hg. The ileum was found to be even less sensitive as pressures above 100 mm. Hg had no effect on the blood-pressure levels.

The response of the blood pressure to distension of the pyloric antrum, pyloric sphincter and duodenum has also been examined in the spinal cat and in cats anæsthetized with chloralose. In the spinal cat a rise of blood pressure

Fig. 2. Decerebrate cat. Tracing to show fall of blood pressure on distension of duodenum. Pressure 10 mm. Hg.

was recorded, but only when higher pressures were used than those usually found necessary to elicit dilatation of the pupil. After section of the splanchnic nerves on both sides, the blood-pressure rise was abolished.

In cats under chloralose anæsthesia the pupil was observed as well as the changes in blood pressure. Distensions of the alimentary canal which produced a slight dilatation of the pupil were usually without effect upon the blood pressure. Higher pressures produced not only a dilatation of the pupil but also a rise in blood pressure. The responses were abolished after section of both pathways.

Action of cocaine

As some regions, for example the pyloric antrum, are closely attached to the posterior abdominal wall by the mesentery, it is possible that the reflex response obtained on distension of the antrum, particularly when high pressures are used, might be due to traction on the nerve fibres supplying the muscle through the mesentery. To test this point we have carried out the following experiments.

In a cat anæsthetized with chloralose, the vagus nerves in the neck were cut, the abdomen was opened and balloons inserted so as to distend the pyloric antrum and the duodenum. After the threshold pressures required to elicit dilatation of the pupil had been recorded, the mesentery attached to the region of the pyloric antrum was painted with a warm 1 p.c. cocaine hydrochloride solution. The mesentery attached to the region of the duodenum which the balloon distended was then painted with a warm saline solution to act as a control. The abdomen was closed and after some 10 min., when the pupils were again constricted, the response to distension was tested. The results are given in Table III.

TABLE III. Cat. Chloralose. Vagus nerves cut in neck. Minimum pressures required to elicit dilatation of pupil.

	Pyloric antrum	Duodenum (Control)
2.45 p.m.	25 mm. Hg	70 mm. Hg
2.50 p.m.	Painted 1 p.c. cocaine	Painted warm saline
3.00 p.m.	100 mm. Hg	75 mm. Hg
3.15 p.m.	95 mm. Hg	50 mm. Hg
4.00 p.m.	50 mm. Hg	55 mm. Hg

It will be observed that after painting the mesentery with a 1 p.c. solution of cocaine, a pressure of approximately 100 mm. Hg was required to elicit the reflex. Little or no change of threshold was observed in the control, and the pyloric antrum was not damaged by the manipulations as its sensitivity increased some 60 min. later. In some other experi-

ments application of 2 p.c. cocaine hydrochloride to the mesentery combined with injection of cocaine into the muscle at the mesenteric border raised the threshold pressure from 30 to over 200 mm. Hg.

The application of cocaine at the mesenteric border of the antrum blocks the nerve impulses from the nerve endings in the muscle of the antrum, and we suggest, therefore, that whereas the reflex response obtained with pressures of 100 mm. Hg or more is due to traction on the nerve trunks, the dilatation of the pupil obtained on distension of the antrum with pressures of less than 100 mm. Hg is due to stimulation of nerve endings in the muscle wall.

It is, of course, possible to contend that the reflex response of the pupil may be brought about through alterations in the blood flow of the part under examination. We would point out that dilatation of the pupil was obtained with low pressures, indeed we have reported experiments where a pressure of 3 mm. Hg was an effective stimulus. Further, after painting the mesenteric border of the pyloric antrum with a 1 p.c. cocaine solution no effect was observed with pressure up to 100 mm. Hg. In this experiment distension would presumably produce similar changes to those produced in the normal experiment. We do not consider, therefore, that changes in blood flow on inflation can account for the reflex response.

Discussion

In the course of this investigation we have used dilatation of the pupil in the cat anæsthetized with chloralose and reflex changes of blood pressure in the decerebrate cat to ascertain the afferent innervation of the mesenteries, stomach, duodenum, jejunum and ileum.

While both methods have proved satisfactory, we consider that dilatation of the pupil due to inhibition of the third nerve nucleus is the more sensitive index of visceral afferent fibres. We would draw attention to the fact that a positive result with either method does not afford evidence of the character of the impulses. We have found that afferent fibres in the vagus and splanchnic nerves innervate the stomach and duodenum, yet we are unable to assign the role played by the afferent impulses in the different nerves.

We have been able to confirm the observations of previous investigators that, while distension of the intestine is an adequate stimulus for sensation, cutting and burning of the wall has no effect. An examination of the results show that we have obtained lower threshold values than have been recorded by a number of authors. At the same time we realize

that our values serve only as an index and do not necessarily give a true picture of the sensitivity of the different regions as the animals were anæsthetized or decerebrated.

On reviewing the work of other authors we have failed to find any report of the volumes of air used to produce the effective pressure. It seemed possible that in the course of the experiment the condition of the viscera might alter considerably, and we therefore decided to calibrate the balloons used and to record simultaneously the volume in c.c. and the pressures in mm. Hg. It will be seen from the results included that while the volumes of air required to elicit a response in the different experiments varied considerably, yet if the pressure required to evoke a response before and after nerve section remained the same, the volumes of air required to produce the effects were approximately equal. It would appear, therefore, that the state of the viscera differed markedly in different animals. The condition or tone of the muscle in any one animal, however, was apparently not appreciably affected by section of either the splanchnic or vagus nerves.

It was reported in a previous communication [Irving & McSwiney, 1935] that section of either the vagus or the splanchnic nerves resulted in an increase in the threshold pressure required to elicit dilatation of the pupil. Subsequent experiments with improved technique have demonstrated, however, that section of one of the afferent pathways does not necessarily involve a decrease in the sensitivity of the viscera.

It seems certain that afferent fibres in the vagus and splanchnic nerves innervate the stomach, pyloric sphincter and duodenum, while afferent fibres in the sympathetic chain are probably the main afferent supply for the jejunum and mid-ileum. The threshold values for the jejunum and mid-ileum are much higher than those for the stomach and duodenum. We have usually failed to obtain any response from the lower ileum even when the intestine was distended with high pressures. There would appear to be a gradient, the intestine becoming less sensitive to distension as the ileum is approached.

We also advance evidence in this paper to show that the gastric and duodenal mesenteries are innervated by the vagus and splanchnic nerves. On the other hand, we were unable to obtain a reflex response on stimulation of the jejunal mesentery when the afferent fibres in the vagus nerves were the sole conducting pathway.

In the course of our experiments the spinal vagus fibres [Harper *et al.* 1935] were usually left intact. As no response was obtained after section of the vagus nerves in the neck and the splanchnic nerves in the

thorax or abdomen, it appeared that the spinal vagus fibres did not form an important afferent pathway from the regions we have examined.

We have not attempted in this investigation to differentiate between the different divisions of the splanchnic nerves. Post-mortem examination has usually shown that in those animals where the splanchnic nerves were cut, all divisions were severed.

Summary

1. Dilatation of the pupil in the cat anæsthetized with chloralose, and reflex changes of blood pressure in the spinal and in the decerebrate cat, and in the cat anæsthetized with chloralose have been used as indices of visceral afferent impulses. These methods do not indicate the character of the sensation.

2. The gastric and duodenal mesenteries are innervated by the vagus and splanchnic nerves while the jejunal mesentery appears to be supplied by the splanchnic nerves. The mesenteries are sensitive to traction.

3. The body of the stomach, pyloric antrum and pyloric sphincter are sensitive to distension and are innervated by afferent fibres in the vagus and sympathetic nerves. The lowest threshold pressure was obtained from the pyloric sphincter.

4. The stomach and duodenum are supplied by afferent fibres from the right and left vagus nerves and from the right and left splanchnic nerves.

5. The jejunum and ileum are much less sensitive to distension than the stomach and duodenum. The right and left splanchnic nerves appear to be the main afferent pathway from these regions.

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A SIMPLE CLOSED-CIRCUIT METABOLISM APPARATUS FOR SMALL ANIMALS

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The closed circuit apparatus to be described is capable of modification and refinement (particularly in the matter of weight) for special purposes, but the way in which it is made must depend largely on the requirements of the user. It will therefore be described in its simplest form, in which it is satisfactory for making determinations of the metabolic rate of small animals over several hours, and for class demonstrations.

The chamber was devised in the first place for following the metabolism of pregnant mice, whose gestation period is 3 weeks. It was felt that metabolic rate determinations lasting only for an hour or so every day would be very short compared with the whole period under consideration, and it was desirable that the animal should be left in a metabolism chamber for 12 hours or more every second day, or every day if necessary, thereby increasing the probability that any changes found would be significant. It was also desired to make three or four determinations simultaneously on different mice. These considerations made the usual types of apparatus unsatisfactory either on the grounds of complexity, expense or multiplicity of measurements.

Principle

Two desiccators are placed together as in Fig. 1, so as to make a single chamber. The mouse is placed in the lower half, and an absorbent for carbon dioxide and water suspended in the upper half. The chamber is closed by a circulating device which connects the two halves externally. As the carbon dioxide and water exhaled by the mouse are absorbed, oxygen is drawn in from a reservoir (e.g. a spirometer) to replace them, thus keeping the composition of the inside air approximately constant.

The increase in weight of the chamber during the experiment gives the amount of oxygen used by the mouse. A method for determining the amount of carbon dioxide given off will be described.

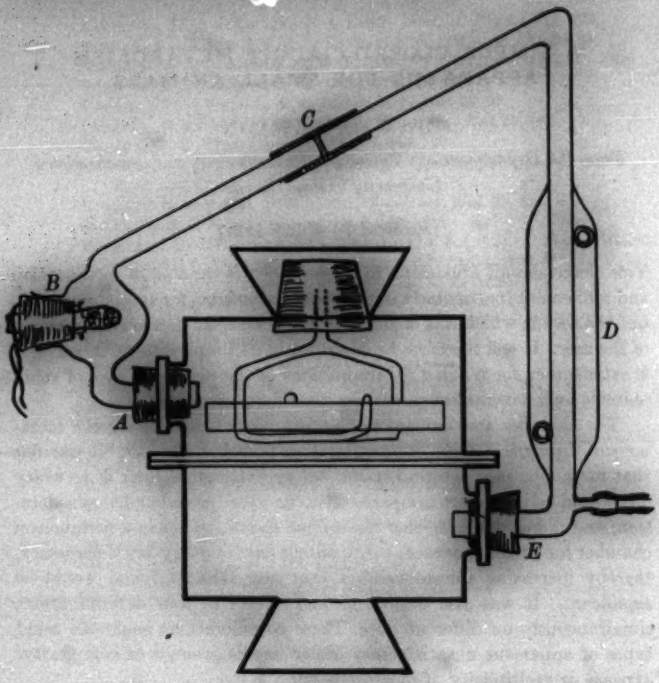

Fig. 1.

The air is kept circulating by the tube *ABCDE*. At *B* is a hot-point, and at *D* a water jacket through which cold water is continually flowing. These create a considerable current of air, whose linear velocity (measured by injecting tobacco smoke through the rubber junction *C* at right angles to the stream) is about 11 cm. per sec. The circulating tube is removed during weighing of the chamber and must therefore be jointed at *C*. It fits easily into the chamber through bungs at *A* and *E*, oxygen being admitted through a side tube near *E*.

Details of apparatus

Suitable desiccators for mice are 4 in. in diameter.

The substance used for absorbing carbon dioxide and water is "soda asbestos" (B.D.H.), a granular product, resembling soda-lime in appearance, but brown in colour. It contains 90 p.c. NaOH and no lime, and dissolves readily in water, leaving a fine brown precipitate (presumably asbestos) which is free from carbonate, even if the original granules have been exposed to CO_2. The great advantage of soda asbestos is that it combines the absorptive power of soda-lime with the solubility of caustic soda, and can therefore be used for the quantitative determination of the CO_2 evolved during an experiment. Caustic soda and potash themselves do not keep the CO_2 percentage within the chamber low enough, while soda-lime is unsatisfactory for quantitative work other than weighing. With soda asbestos, after a mouse has been in the chamber as long as 15 hours, the percentage of CO_2 is only 0·5, while if a complete diaphragm of wire gauze is used instead of a simple glass tray to support the soda asbestos, the CO_2 can be kept down to about 0·2 p.c. Iron wire gauze oxidizes, and the most satisfactory substitute would probably be a sintered glass filter, but this has not been tried. There is no evidence that 0·5 p.c. CO_2 appreciably affects the metabolic rate, and the mice appear perfectly normal.

The glass tray (e.g. a Petri dish) rests in a frame of thin glass tubing (Fig. 1). The thicker vertical tube from which this frame is suspended can be thrust into a bored cork which fits into the upper desiccator as shown. It is important that the edge of the tray should be close enough to the side of the desiccator to prevent the mouse from climbing. The tray must also be at such a height that the upper desiccator slides easily off the lower.

The oxygen is originally transferred from a cylinder to a reservoir, e.g. a spirometer, from which it is supplied to the apparatus over anhydrous $CaCl_2$. Between the $CaCl_2$ and the chamber is interposed a sulphuric acid valve through which the oxygen bubbles slowly. If this does not increase in weight during the experiment it is assumed that the oxygen passing into the chamber is dry. It is important to realize that this oxygen contains about 2 p.c. of nitrogen, so that if the chamber contains atmospheric air at the beginning of an experiment, the percentage of oxygen will ultimately fall to a low level. It is therefore advisable in practice to start the experiment with the chamber full of oxygen instead of air. Sufficient weight is applied to the top of the spirometer to overcome the resistance of the H_2SO_4 valve.

Bungs to close the chamber at *A* and *E* should be kept with the chamber, which must be airtight during weighing when the circulating tube has been removed.

The hot-point was made by winding resistance wire round a piece of clay pipe stem and fixing it with fireproof cement. This also served to seal the outer end of the pipe stem which was then varnished. The resistance of the hot-point was 10 ohms, and it was heated from the mains (220 V.) in series with a 150 W. lamp.

Procedure

The hot-point is switched on half an hour before the experiment begins to ensure that the walls of the circulating tube are free from moisture when it is used.

A mouse is placed in the lower compartment of the chamber, and the greater part of a weighed quantity of soda asbestos introduced into the tray. The two halves are now placed together, but before stoppering the

chamber is rapidly flushed out with oxygen. It is then stoppered, the time being taken, and weighed with its contents. After this, the stoppers are removed and the circulating tube quickly placed in position. Oxygen is then seen bubbling steadily through the H_2SO_4 trap into the chamber, which can be left. It may be placed in a constant temperature bath if necessary. The residue of the soda asbestos is weighed, dissolved in distilled water and made up to 100 c.c. The CO_2 content of the solution (shaken before sampling so as to get an even suspension of the precipitate) is determined by van Slyke's (manometric) method.

It is unnecessary to describe the technique of this determination, since it is much the same as that described in the original paper of van Slyke & Neill [1924].

The reagents, however, must be chosen with regard to the alkalinity of the soda-asbestos solution. In the experiments described below 0·1–0·4 c.c. of 12*N* H_2SO_4 and 1 c.c. of 10*N* KOH were used for the evolution and reabsorption respectively of the CO_2 in 1 c.c. of soda-asbestos solution.

At the end of the experiment the circulating tube is rapidly replaced by the stoppers, the time taken, and the chamber and its contents weighed. The increase in weight represents the oxygen used. The chamber is then opened, the soda asbestos dissolved and made up to 1 l. with distilled water, and the CO_2 content of the solution determined. The volume of CO_2 produced by the mouse can now be simply calculated. The following protocol makes the method clear:

Weight of:

(*a*)	Weighing bottle (*WB*) + soda asbestos	100·12 g.
(*b*)	Chamber + mouse + some soda asbestos (SA_1) (before exp.) ...	2264·70 g.
(*c*)	$a - SA_1$	66·16 g.
(*d*)	SA_1 $(a - c)$	33·96 g.
(*e*)	*WB* empty	47·35 g.
(*f*)	Soda asbestos residue (SA_2) $(c - e)$	18·81 g.
(*g*)	Chamber and contents after exp.	2266·72 g.
(*h*)	Oxygen used $(g - b)$	2·02 g.
		= 1414 c.c.
(*k*)	CO_2 content of SA_2	96·7 c.c.
(*m*)	Original CO_2 content of SA_1 $(k.d/f)$	173 c.c.
(*n*)	CO_2 content of SA_1 after exp.	1374 c.c.
(*p*)	CO_2 evolved $(n - m)$	1201 c.c.
(*q*)	Respiratory quotient	0·85

Duration of exp. = 18 hours.

Accuracy

The weighings in the foregoing experiment were accurate to the nearest 10 mg., and as the weight of oxygen used was obtained by difference, there is a possible error of ±0·01 g. in 2·02 g., i.e. ±0·5 p.c. The carbon dioxide measurement is much more accurate than this. The greatest source of error in the van Slyke method is in measuring the sample into the apparatus, but for this experiment it is sufficient if the

final readings of the manometer for determinations of successive samples agree to the nearest mm. Hg, and this degree of accuracy is easy to attain. The error in the total CO_2 content of the spent soda asbestos (SA_1) in the foregoing experiment is only $\pm 0{\cdot}1$ p.c. so far as the estimation is concerned. In subtracting from this the amount of CO_2 originally present, a figure is used which depends on weights, but if the protocol is examined it will be seen that the error from this source is less than 0·1 p.c. in the figure (173 c.c.) to be subtracted. Since this is of the order of one-tenth of 1374 c.c. it can introduce no error into the final result.

Errors which cannot be estimated numerically may be introduced during the various operations. In attaching the circulating tube after the first weighing there is an opportunity for CO_2 and water vapour to escape, and in detaching it preparatory to the final weighing there is also the possibility of loss, both through the bung holes and in the circulating tube itself. The percentage of CO_2 in the chamber is never more than 0·5 p.c., and the total amount present is therefore less than 5 c.c. It is unlikely that the small proportion of this escaping during the rapid change from bungs to circulating tube and vice versa would be outside the experimental error of the weighing or even of the CO_2 estimation. The same type of reasoning applies to water vapour; which would be rather less in weight than, though about twice the volume of, the CO_2.

Corrections

In some circumstances it may be necessary to correct for the difference in composition of the gas in the chamber at the first and second weighings. First, some of the oxygen is replaced by nitrogen. In the example given above, the 2 litres of oxygen used would have carried into the chamber about 50 c.c. of nitrogen, and at the second weighing this is weighed instead of a corresponding volume of oxygen. The chamber is therefore about 9 mg. too light. Secondly, if 0·5 p.c. (5 c.c.) of CO_2 is present at the time of the second weighing, this displaces completely an equivalent volume of oxygen weighing about 7 mg. The humidity inside the chamber stays approximately constant between 40 and 50 p.c. saturation for at least eighteen hours, owing to the hygroscopic nature of soda asbestos, and provided a few drops of water are placed in the bottom of the chamber at the beginning of the experiment, no error is introduced. (The humidity was estimated by the readings of a paper hygrometer (Pastorelli & Rapkin) small enough to go inside the chamber, and freshly calibrated over the appropriate range. It is highly probable, though difficult to prove, that the variations of 10 p.c. or so in the humidity readings were

due to the mouse occasionally breathing into the instrument.) The total correction, therefore, in an experiment lasting 18 hours is not more than 16 mg., and for comparative purposes would be unnecessary. It is easily made by measuring the nitrogen content of the oxygen used, the final percentage of CO_2 in the chamber, and the volume of the chamber and circulating tube.

Temperature

The empty chamber has been tested at temperatures between 17 and 37° C., and always takes the temperature of its surroundings within 1° C. A pair of thermocouples were connected to a recording galvanometer. One couple was placed in the chamber, the other in an adjacent flask open to air, or, if the chamber was in a water bath, in the water of the bath. At all temperatures tested, the galvanometer recorded a straight line within 1° C. of the zero line obtained when both thermocouples were in the same medium. The greatest deviation was when the air outside was 17·3° C., and that inside 18° C. At other temperatures the record was for all practical purposes on the zero line. An experiment (with an empty chamber) lasting overnight yielded a straight line record although the maximum and minimum thermometer showed a variation between 20·0 and 23·3° C. An experiment in which the outside temperature was raised through 3° C. in 30 min., disclosed a lag in the equilibration of the chamber whose temperature had risen less than 1° C. In the next half-hour the temperature of the medium rose a further 1° C., and that of the chamber came to within 0·5° C. of equilibrium. The chamber is therefore insensitive to rapid fluctuations of temperature.

The flow of water in the condenser was altered between 0·15 and 1·3 l. per min. without affecting the temperature of the inside of the chamber.

Alcohol check

Alcohol was burnt in the chamber to check the chemical accuracy of the method, and a typical protocol is given below. The circulating tube was omitted from the apparatus to allow the insertion of a bung at *A* carrying an electro-cautery. The cautery was in contact with the wick (a single strand) of a small spirit lamp of about 3 c.c. capacity, and a neck of 2 mm. diameter. This was sufficient to support the wick and yet leave an opening alongside it, thus ensuring equality of pressure inside and outside the lamp. Many other types of lamp and other devices for burning a known quantity of alcohol were tried, but this was the only one which worked. When the cautery was switched on from outside, the lamp lit

and burnt steadily with a minute blue flame. Too large a flame caused so much expansion in the chamber as to delay the entry of oxygen until after it had been extinguished, and relighting was useless on account of the loss of alcohol meanwhile by evaporation. In order to make sure that the lamp burnt dry, a certain volume of oxygen had to be admitted in the first place, otherwise the percentage of oxygen became too small during the experiment to support combustion (temporary replacement by N_2, H_2O vapour and CO_2). Once a suitable lamp had been made, this was the greatest obstacle encountered, for too much oxygen caused an explosion on heating the cautery. It was found that if 250–300 c.c. oxygen were allowed to flow from the reservoir through the unstoppered chamber, a suitable gas mixture was obtained, but this was never analysed. The following record of a check is arranged to indicate the procedure, which is more complicated than in a metabolism experiment, as well as to present the result.

(*a*) Weight chamber	2212·91 g.
(*b*) Weight weighing bottle (*WB*) + soda asbestos (*SA*)	86·59 g.
(*c*) Weight lamp, after burning out preliminary charge of alcohol ...	5·68 g.
(*d*) Weight lamp + alcohol	7·37 g.
(*e*) Weight alcohol (*d* − *c*)	1·69 g.
Lamp and some soda asbestos (SA_1) transferred to chamber, through which 300 c.c. O_2 allowed to run. Chamber closed. Lamp ignited by red-hot cautery. Lamp burnt for 4½ hours.	
(*f*) Weight (*WB* + *SA*) − SA_1	58·18 g.
(*g*) Weight SA_1 (*b* − *f*)	28·41 g.
(*h*) Weight chamber + contents before combustion (*a* + *d* + *g*)	2248·69 g.
Remaining soda asbestos (SA_2) dissolved and made up to 100 c.c. in distilled water.	
(*k*) Weight SA_2 (*f* − 47·34)	10·84 g.
(*m*) CO_2 content SA_2 (by van Slyke's method)	43·28 c.c.
(*n*) CO_2 content SA_1 before combustion (*m.g*/*k*)	113 c.c.
(*p*) Weight chamber + contents after combustion	2252·18 g.
(*q*) Weight O_2 used during combustion (*p* − *h*)	3·49 g. = 2443 c.c.
Chamber opened, SA_1 dissolved and made up to 1 l. in distilled water.	
(*r*) CO_2 content SA_1 after combustion (by van Slyke's method) ...	1732 c.c.
(*s*) CO_2 evolved during combustion (*r* − *n*)	1619 c.c.
(*t*) Respiratory quotient of combustion (*q*/*s*)	0·663
(*u*) Theoretical amount O_2 required by combustion of 1·69 g. of 98·8 p.c. ethyl alcohol	3·48 g. = 2436 c.c.
(*v*) Theoretical volume CO_2 evolved during combustion of 1·69 g. of 98·8 p.c. ethyl alcohol	1626 c.c.
(*w*) Theoretical respiratory quotient	0·667

By connecting the water condensers and the hot points in series and oxygen supply tubes in parallel any number of these metabolism chambers can be used simultaneously.

Summary

A simple closed metabolism apparatus is described, in which both oxygen consumption and carbon dioxide output can be measured. In its present form it is most suitable for small animals and for experiments lasting over several hours. The circulation of air inside the chamber is maintained without any machinery. Several of these chambers can be connected to the same oxygen reservoir and used simultaneously.

I wish to thank Prof. Lovatt Evans for suggesting the possibility of heat as a circulating agent, and for his continued help and advice. My thanks are also due to Mr J. B. Brooksby and Mr J. L. Parkinson for their help in several of the experiments.

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THE CHOLINESTERASE IN SYMPATHETIC GANGLIA

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The action of acetylcholine as the transmitter of excitation at preganglionic synapses and at motor endings in voluntary muscle, for which evidence has been produced during the past few years, must be of a different kind from its earlier recognized functions as the transmitter of peripheral parasympathetic effects. There is no evidence that, under normal conditions, the effect of an impulse in a preganglionic or voluntary motor nerve fibre spreads beyond the ganglion cells or muscle fibres with which it makes direct synaptic connexion, or that a single such impulse normally excites more than a single wave of excitation in postganglionic nerve or in muscle fibre. The effect is thus subject to spatial and temporal limitations which do not apply to those earlier associated with chemical transmission. The nature and the probable mechanism of such limitations have been discussed in the case of the ganglion by Feldberg & Gaddum [1934] and Feldberg & Vartiainen [1934] and in that of the voluntary muscle by Dale *et al.* [1936], and Brown *et al.* [1936], who have recognized the necessity of provision for the very rapid removal of the liberated acetylcholine, within the very brief span of the refractory period of the ganglion cell or muscle fibre. A local concentration of the specific cholinesterase on surfaces at the nerve endings was an obvious possibility.

If the method for removal of acetylcholine were really of this kind, it ought to be possible by the use of eserine to delay the disappearance of acetylcholine, and to observe a corresponding effect on the transmission of the excitatory process. Brown *et al.* [1936] and Bacq & Brown [1937] have in fact demonstrated that eserine, and various synthetic substances having an analogous depressant action on the cholinesterase, cause the voluntary muscle fibre to give a repetitive response to a single nerve

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impulse, in exact accordance with this expectation. In the case of the ganglion cells no such clear evidence is yet available; the picture is complicated by the fact that eserine, in more than minimal concentration, appears to exercise a depressant action on the response of the ganglion cells to any form of stimulus; and there is not yet any clear evidence as to whether the persistence of acetylcholine in contact with a ganglion cell beyond the refractory period would, indeed, as in the case of the muscle fibre, evoke a repetitive response.

There is, accordingly, definite though indirect evidence in favour of a concentration of cholinesterase at the motor nerve endings in voluntary muscle, but, in the case of ganglionic synapses, only such evidence as analogy with these motor nerve endings affords. It seemed desirable to attempt to obtain evidence of a more direct kind. It was already known that the acetylcholine, obtainable by extraction from an organ supplied with cholinergic nerve fibres, and presumably held during life in readiness for release by the arrival of impulses at the nerve endings, disappears when the nerve fibres in question are cut and allowed to degenerate. This was shown by Engelhart [1931], in the case of the ciliary body and iris of the cat's eye, and by Brown & Feldberg [1936], in that of the superior cervical ganglion. The "depot" holding the acetylcholine ready for release being thus definitely associated with the integrity of the nerve endings, it was natural to enquire whether the cholinesterase, required for its rapid removal, would show a similar association and dependence. In the case of the voluntary muscle it would be difficult to obtain direct evidence on the point, since the nerve endings are widely scattered in a large mass of muscle, and even a high concentration of the esterase at each would probably make but a small contribution to the yield from the whole tissue. In the case of the ganglion, on the other hand, the synapses are in much greater abundance in relation to the total mass, and an attempt to obtain direct evidence seemed the more desirable, in view of the lack of it from the action of eserine. I have accordingly made a number of comparisons of the cholinesterase obtainable by extraction from normal sympathetic ganglia, and from corresponding ganglia deprived of synaptic endings by degenerative section of their preganglionic fibres.

Methods

The ganglia chosen for examination were the superior cervical ganglia of the cat, either normal, or preganglionically denervated by aseptic division of the cervical sympathetic nerve at an earlier date. In some cases the adjacent ganglion of the vagus trunk was examined as a control.

A few experiments were also made on the normal ganglion of the horse. Adult cats were used and the aseptic section of the cervical sympathetic nerve was carried out under ether anæsthesia, periods of 4–31 days being allowed for degeneration. About a centimetre of the nerve was removed at the operation, at a site remote from the ganglion, which was not exposed or otherwise disturbed.

The extracts from the ganglia, for determination of the esterase content, were made with Tyrode solution, if the final assay of acetylcholine was to be made on a strip of rabbit's intestine, or with Ringer (frog) solution, if the rectus abdominis muscle of the frog was to be the test object. The cats were killed before dissection of the ganglia, which in some cases were briefly perfused from the carotid arteries with Locke's solution before removal, to free them from traces of blood. Extracts made from cat's blood, however, showed that, weight for weight, its esterase activity, in the time of incubation allowed and at the dilution used, was negligible in comparison with that of the ganglionic tissue, so that its removal by perfusion was an unnecessary precaution. Each ganglion, dissected as cleanly as possible, was dried superficially with filter paper and weighed. It was then put into a small mortar, and the extracting saline fluid was added in the proportion of 0·1 c.c. for each mg. of ganglion. The ganglion was then minced carefully in the fluid with fine scissors, and ground after further addition of 0·5 g. of washed silver sand. Tissue and sand were then thrown down by the centrifuge, leaving a water-clear supernatant extract. The method of measuring the esterase activity was based on that used by Plattner & Hintner [1930], 0·5 c.c. of the clear extract being mixed with 0·5 c.c. of a 1 in 10^4 solution of acetylcholine chloride (Roche), 1 c.c. of *M*/15 phosphate buffer of *p*H 7·8 (or 7·5) being added to adjust the reaction, and the mixture then allowed to stand at room temperature for 15–45 min. At the chosen moment the esterase action was stopped by adding 0·5 c.c. of a 10 p.c. solution of trichloroacetic acid. Control samples of acetylcholine, made up to the dilution and reaction with buffer solution, but without ganglion extract, and allowed to stand for comparable times at the same temperature, showed no perceptible loss of activity.

Immediately before the assay of such a reaction mixture for the unhydrolysed acetylcholine, it was brought to approximately neutral reaction by careful addition of *N*/5 NaOH, and then made up to 5 c.c. with the saline solution (Tyrode or frog Ringer) in which the test was to be conducted. Each mixture, as thus finally diluted, accordingly represented an original acetylcholine content of 1 in 10^5. The tests used

were those on the rectus abdominis of the frog sensitized by eserine [Chang & Gaddum, 1933], and on the loop of rabbit's jejunum [Dale, 1914].

RESULTS

Table I shows the comparative figures obtained for the destruction of acetylcholine under the conditions above described, by extracts from normal sympathetic ganglia, of the corresponding ganglia after pre-

TABLE I. The esterase activity of normal and preganglionically denervated cervical sympathetic ganglia, and of normal vagus ganglia, in cats

	Weight of ganglia in mg.				Percentage of acetylcholine destruction			
No.	Normal	Decentralized	Vagus	Time of incubation min.	Normal	Decentralized	Vagus	Days since decentralization
1	—	—	—	35	91 98	—	—	—
2*	15·24 17·90	—	—	30	96 96	—	—	—
3	—	—	—	35	95 95	—	—	—
4	16·86 17·94	—	10·6 12·2	40	96 94	—	67	—
5	18·44 16·72	—	10·96 7·94	35	98 99	—	60	—
6	15·49 18·24	—	12·58 8·40	45	90	—	50	
7	12·32 13·10	—	11·46 9·76	15	95	—	30	—
8	21·96 21·80	—	12·54 12·14	30	90	—	40	—
9	27·24	27·14	—	15	90	65	—	14
10	13·52	10·40	17·52	40	100	67	72	14
11	15·36	18·16	17·64	30	56	15	20	17
12	12·98	9·56	16·94	40	83	10	35	13
13	16·46	14·58	—	35	80	20	—	24
14	14·56	12·88	10·34 9·10	40	83	55	46	31
15	17·94	17·78	11·72 8·96	15	75	40	35	8
16	11·98	11·90	8·00 8·16	35	70	25	40	9
17	11·90	13·42	10·36 8·12	35	83	15	55	4
18	13·16	12·70	11·28 7·84	35	95	50	75	15
19	7·30	6·92	—	—	75	40	—	13

* If two figures are given in one column, the ganglia of the two sides have been weighed, or assayed for esterase, separately.

ganglionic denervation and of normal vagus ganglia. It will be noticed that the extracts of normal sympathetic ganglia invariably cause a large breakdown of acetylcholine in the time allowed for contact between enzyme and substrate. That this is not due to any unspecific action, but to the presence of cholinesterase can be proved by the fact that addition of eserine in a final concentration of 1 in 10^5 completely inhibits this destruction (see tracings 2 and 3 in Fig. 1). The somewhat varying rate of esterase activity observed in different experiments may be due to variations of the room temperature at which the samples were allowed to stand. The normal right and left superior cervical ganglia exhibit in every case an almost identical esterase activity (see Exps. 1–5 in Table I), the biggest difference being 7 p.c., which is not significant, in consideration of the difficulty of accurate determination of the minute amounts of acetylcholine which remain in these experiments.

On the other hand, comparison with the normal vagus ganglia in Table I shows that the latter have invariably less esterase activity than the sympathetic ganglia of the same animal, although the presence of that enzyme in normal vagus ganglia is quite unmistakable. This comparison in itself suggests that the excess of cholinesterase in the case of the sympathetic ganglion must be connected with the presence of synaptic nerve endings; for, apart from absence of synaptic interruption of the nerve fibres passing through the vagus ganglion, there is no obvious reason for expecting such a chemical difference between the two ganglia. This connexion between the presence of synaptic endings and the excess of esterase is more clearly revealed by the fact that the difference disappears after degeneration of the preganglionic fibres. It will be seen, in Table I, that the preganglionically denervated sympathetic ganglia yield not more, but in most cases even less, esterase than the corresponding vagus ganglia. In any case they stand, with the vagus ganglia, in sharp contrast to the normal sympathetic ganglia. Complete histological degeneration of the preganglionic fibres seems, however, not to be necessary to produce the loss of esterase, since, in Exp. 13, the difference between normal and denervated ganglia is well established as early as 4 days after division of the cervical sympathetic. The same difference in esterase activity, between normal and decentralized cervical sympathetic ganglia, can also be observed after cutting all the connexions of the stellate ganglion, except that with the cervical sympathetic nerve. Exps. 16 and 17 in Table I were made with the ganglia of cats in which this operation had been carried out on one side from a dorsal incision, after the method of H. K. Anderson [1904].

Figs. 1 and 2 illustrate the methods of estimation used in obtaining the data for Table I. In Fig. 1 the record is from an isolated loop of rabbit's jejunum. Mixtures of ACh. with ganglion extracts stood in all

Fig. 1. The evaluation of esterase activity in normal and decentralized sympathetic ganglia using rabbit's jejunum as test. (For details see text.)

Fig. 2. The same as Fig. 1, using the frog's eserinized rectus abdominis. (For details see text.)

cases for 40 min. at room temperature. The additions to the bath corresponding to the numbers on the tracing may be classified as follows:

(1) Untreated ACh.: $1=1{\cdot}5\gamma$; $2=2\gamma$; $6=2\gamma$; $7=1{\cdot}8\gamma$; $8=1\gamma$; $10=1{\cdot}5\gamma$; $11=1{\cdot}7\gamma$; $12=1{\cdot}9\gamma$; $14=2\gamma$.

(2) ACh. + extract of normal sympathetic ganglion: 9 and $13=10\gamma$.

(3) ACh. + extract of denervated sympathetic ganglion: $4=3\gamma$; $5=2\gamma$.

(4) ACh. + extract of normal ganglion + eserine (10^{-5}): $3=2\gamma$.

It will be seen that the effects at 9 and 13 are slightly greater than that at 11 ($1{\cdot}7\gamma$), and less than that at 12 ($1{\cdot}9\gamma$). It may be calculated, therefore, that the 10γ originally present in the dose of mixture given at 9 and 13 has been reduced to $1{\cdot}8\gamma$, i.e. a destruction of 82 p.c. by the

extract from the normal ganglion. Similarly, the effects at 5 and 7 being equal, the dose of 2γ in the mixture given at 5 has been reduced only to 1·8γ, i.e. a destruction of only 10 p.c. by the extract from the denervated ganglion. The fact that the effects at 2 and 3 are equal proves that eserine completely inhibits the destructive effect of the extract from the normal ganglion.

Fig. 2 shows a similar comparison made on the isolated rectus abdominis of a frog, sensitized with eserine. The mixtures were in this case left at room temperature for 35 min. The following are the doses represented:

(1) Untreated ACh.: 1 = 1γ; 4 = 0·08γ; 5 = 0·09γ; 7 = 1γ; 8 = 1·2γ; 9 = 1·4γ.

(2) ACh. + extract of normal sympathetic ganglion: 6 = 9γ.

(3) ACh. + extract of denervated sympathetic ganglion: 2 = 2γ; 3 = 1γ.

Since the effect at 6 is equal to that at 8, 9γ has been reduced to 1·2γ by the action of the extract from the normal ganglion, i.e. a destruction of 86 p.c. Similarly, the effect at 3, being equal to that at 4, 1γ has been reduced to 0·08γ by corresponding treatment with extract from the denervated ganglion, i.e. a destruction of only 20 p.c.

Some quantitative interpretations

I have not made any attempt at a strictly quantitative determination of the relative losses of esterase, represented by the observed reductions in the loss of acetylcholine. It would be necessary for such a purpose to carry out many determinations at different time intervals, so as to estimate the rate of destruction in each mixture. The quantities of material at my disposal forbade any attempt at an estimate of this kind. In order to obtain some idea, however, of the amount of esterase disappearing with denervation, I made some control experiments with extracts from normal sympathetic ganglia diluted to different degrees. Thus, from the extract of a normal sympathetic ganglion, which, in Exp. 5, Table I, destroyed 98 p.c. of the added acetylcholine in a given period, 1 : 2 and 1 : 4 dilutions were made and kept at the same temperature with the same initial concentration of acetylcholine. The results showed a 77·5 p.c. destruction with the 1 : 2 dilution, and a 55 p.c. destruction with the 1 : 4 dilution. If these relations are plotted, a graph drawn through these three points and the point of origin enables a rough estimate to be made of the reduction of the yield of esterase consequent, in each experiment, on preganglionic denervation. Thus in Exp. 18, when the extract

from the normal ganglion destroys 95 p.c. and that from the denervated 50 p.c. of the ACh. added, we can estimate that about 75 p.c. of the esterase normally present in the ganglion has disappeared with denervation. It seems probable that the ratios shown in most experiments represent a larger proportional disappearance than this, and only in a few, such as Exps. 9 and 14, is there reason to suspect that the loss of esterase is smaller, though still probably well over 50 p.c.

It should be noted, in the next place, that this loss of a large proportion of the esterase originally present is accompanied by, at most, a small and variable loss in the weight of the ganglion. Exps. 1–8 show that the ganglion of one side does not furnish an accurate control for the weight of the other. If the average weight of the cervical sympathetic ganglion is separately calculated for the two sides, from the figures of Exps. 1–8, the weights obtained are R 17·01 and L 17·56 mg. respectively. On the average the left ganglia are the heavier. In my experiments the side on which the nerve was cut was always the right. If similar calculations are made for the average weights of the (left) normal superior cervical ganglion and the denervated ganglion of the other (right) side in Exps. 9–19, the weights obtained are 14·67 and 14·13 mg. respectively. The apparent difference is therefore of no significance. If any loss of substance has occurred it must have been due to some constituent of the ganglia enormously richer than the remainder in cholinesterase, since a reduction in weight so small as to be doubtful may be attended with a loss of three-quarters or more of the esterase. This deduction enables us to examine certain possibilities more closely. When the preganglionic fibres are cut, their intraganglionic extensions may be assumed to degenerate down to their synaptic endings. It seemed desirable, therefore, to investigate such fibres in their preganglionic course. For this purpose in Exps. 1 and 5 the cervical sympathetic nerves of both sides were cleanly dissected, and the right and left nerves were weighed and extracted together in each case. In Exp. 1 the weight of preganglionic nerve thus obtained was 20·96 mg., and in Exp. 5, 26·32 mg. Extracts were made in the same proportions as from the corresponding ganglia and incubated with the same proportion of acetylcholine for corresponding tissues. The nerve extract from Exp. 1 showed a 78 p.c. destruction, that from Exp. 5 a 62·5 p.c. destruction of the added acetylcholine in the time allowed. It will be seen that in comparable periods the ganglion extracts destroy so much greater a proportion of acetylcholine that, from the data given above, it may fairly be concluded that the ganglionic tissue as a whole yields upwards of twice as much cholinesterase as the same weight of

preganglionic nerve. While, therefore, it is of some interest to find that the preganglionic nerve contains a significant amount of esterase, just as Barsoum [1935] found that it contained a rather large proportion of acetylcholine, it is quite certain that the loss of a large proportion of the cholinesterase from the ganglion, after the preganglionic fibres are cut, cannot be due to the degeneration of intraganglionic fibres having esterase in as small a proportion as is found in their extraganglionic portions. At some point of their intraganglionic course, therefore, these fibres must acquire a much larger store of esterase, as well as of acetylcholine [Brown & Feldberg, 1936], than they contain before entering the ganglion. The most probable location for the concentration of both appears to be that which has already been suggested, by theoretical considerations of the nature of the synaptic transmission by acetylcholine, namely, at the synaptic endings of the preganglionic fibres.

Summary

1. The normal cervical sympathetic ganglion of cats contains a considerable amount of cholinesterase.

2. The concentration of that enzyme in the ganglion is considerably (probably about 3 times) higher in the sympathetic ganglion than in the normal vagus ganglion or in the cervical portion of the sympathetic nerve.

3. This excess of esterase disappears completely after section of the preganglionic fibres of the sympathetic ganglion, even before these fibres can have completely degenerated.

I wish to thank Sir Henry Dale and Dr G. L. Brown for suggestion and helpful advice in this investigation.

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THE ACTIONS OF ACETYLCHOLINE ON DENERVATED MAMMALIAN AND FROG'S MUSCLE

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Until recently, the only known responses of muscle to acetylcholine have been regarded as contractures, that is, in the words of Gasser [1930], an involvement of the normal contractile mechanism of the muscle "in which tension, heat and lactic acid are produced and the active portion of the muscle becomes negative. Only conduction of the mechanical response and a wave-like action potential are missing." Brown *et al.* [1936] have, however, been able to show that the close arterial injection of acetylcholine in relatively low concentrations can evoke quick, twitch-like responses from normal mammalian muscle, and these responses have been shown to be short asynchronous tetani associated with an outburst of diphasic, conducted action potentials [Brown, 1937*b*]. The slow contracture of denervated mammalian muscle in response to minute doses of acetylcholine has long been known, but Brown *et al.* drew attention to an additional phenomenon, which had hitherto escaped notice. They observed that certain doses of acetylcholine injected arterially produced in denervated muscle "a double effect, consisting of an initial, sharply topped contraction, followed by a secondary, slow contracture". This suggested that part, at least, of the reaction of denervated muscle might be identical with that of normal muscles.

If this was so, it was obvious that the reactions of frog's muscle to acetylcholine would repay further investigation, using methods of arterial injection as like as possible to those used in the mammal. There is already sufficient evidence to suggest that acetylcholine might be capable

of setting up propagated responses in frog's muscle in addition to the well-known contracture, first described by Riesser and Neuschloss [1921]. Langley [1907–14] in a series of papers has established that nicotine when applied to the neural region of a frog's muscle can produce brief twitches and short tetani, which are conducted throughout the muscle, quite apart from the slow contracture which spreads only slowly through the muscle substance. If this is true of nicotine, it appeared likely that it would apply also to acetylcholine, since its activity on normal mammalian muscle [Bacq & Brown, 1937] and on denervated mammalian and frog's muscle [Dale & Gasser, 1926] was of a nicotine-like character. More direct evidence on this point was, in fact, already to be found in papers by v. Neergaard [1924] and by Schäffer & Licht [1926*b*], who described fibrillary twitchings in frog's muscles and fine oscillations in the electromyogram when acetylcholine was injected into the Ringer's solution perfusing them; but in neither case did the authors apparently appreciate the significance of the effects they described.

Throughout this paper, the use of the terms contraction and contracture, in so far as they imply any specific type of reaction, has been avoided. The descriptive terms "quick phase" and "slow phase" of the double reaction of cat's muscle have been extended, though admittedly with less aptness, to cover the description of the responses of frog's muscle. The only evidence accepted for propagated responses in the muscle fibre is the occurrence of rapid, diphasic action potentials. It must be made clear that no attempt has been made to record slow potential changes between active and passive portions of a muscle; indeed, they have been deliberately excluded by using small coupling condensers in the amplifier. There is ample evidence that such potentials occur with slow changes of tension [Riesser & Steinhausen, 1922; Schäffer & Licht, 1926*a*, *b*]; but their distinction from propagated action potentials is made abundantly clear by the experiments of Bishop & Gelfan [1932], who point out that a localized contracture of a single fibre may produce an action potential, but that this is monophasic and only recordable when one electrode is actually on the spot at which the contracture occurs.

Some observations have been made on the effects of adrenaline, eserine, curarine and atropine on the responses of muscles to acetylcholine. A preliminary account of these experiments has appeared in this *Journal* [Brown, 1937*a*].

Methods

Mammalian muscle. For the experiments on denervated mammalian muscle, cats were used. Under ether anæsthesia and with aseptic precautions, the sciatic nerve on one side was divided high in the thigh. Degeneration was allowed to proceed for between 4 and 27 days in different experiments. After sufficient time had elapsed, the animals were anæsthetized with ether and either decerebrated, rendered spinal, or given sufficient chloralose intravenously to maintain anæsthesia after withdrawal of the ether. The gastrocnemius on the operated side was then prepared for arterial injection of acetylcholine into the tibial artery. The method of preparation and injection has already been described [Brown *et al.* 1936; Bacq & Brown, 1937]. All arterial injections were made in a volume of 0·5 c.c. Electrical records of the response to the injection of acetylcholine were taken with concentric needle electrodes, amplifier and oscillograph, together with a simultaneous isometric record of the contraction of the muscle [Brown, 1937*b*].

In many experiments records were taken, on a smoked drum, of the responses to acetylcholine injection and to direct stimulation of the muscle. The stimuli were break induction shocks, timed by a Lewis contact breaker and led to the muscle through the fixing drill in the condyles of the femur and a wire sewn into the tendon, below the insulated hook attaching it to the myograph.

Frog's muscle. The method used for the frog experiments was, in essentials, the same as that for the cat. The Hungarian *Rana esculenta*, weighing between 100 and 200 g. was found to be large enough to enable close arterial injections to be made into the gastrocnemius. The brain and spinal cord having been destroyed with a steel wire, the skin over the dorsum of the thigh was opened and the incision continued to the knee-joint. Here a number of large cutaneous vessels, arising from the sciatic artery, must be tied and cut before continuing the incision over the gastrocnemius. The Achilles tendon was then severed and the gastrocnemius raised from the tibia and fibula, exposing the peroneal and tibial arteries, which, together with the peroneal and tibial nerves, were tied distal to the origin of the gastrocnemius; the remaining cutaneous and muscular branches of the tibial and peroneal arteries, other than those supplying gastrocnemius, were then ligated, and the sciatic nerve was cut high in the thigh and freed for stimulation. The sciatic artery was then tied about 2 cm. proximal to the origin of gastrocnemius and a fine cannula, made from a hypodermic needle, was inserted and tied in as

near as possible to the bifurcation of the artery. Through this cannula the muscle was perfused at a slow rate with Ringer's solution. Injection of ink into the cannula showed that the perfusion was confined almost entirely to the gastrocnemius, there being only a very faint staining of the muscles immediately proximal to the knee joint.

The composition of the Ringer's solution used in the perfusion experiments was as follows: NaCl 7 g., KCl 0·14 g., $CaCl_2$ 0·12 g., $NaHCO_3$ 0·2 g., distilled water to 1 litre.

Acetylcholine injections were made by interrupting the perfusion, filling the butt of the needle cannula with the solution to be injected, and attaching a syringe carrying 0·1 c.c. of the solution. With this volume, injections can be made with considerable velocity, without any danger of rupturing the delicate vessels. The acetylcholine dilutions were made in Ringer's solution immediately before each injection. The diluting solution alone, injected in a like manner, evoked no response from the muscle.

In a few of the later experiments it was found possible to insert a cannula into the tibial artery of the frog and to perform the whole experiment on the decerebrate animal with the circulation intact. This provides a very satisfactory preparation, which has the advantage of not developing œdema as the experiment proceeds.

Action potentials and optical isometric myograms were recorded in the frog experiments with the same apparatus as was used for the cat. The most satisfactory leads for action potentials were found to be from belly to tendon, by means of silver pins thrust into the muscle. No electrical screening, beyond that provided by the metal recording table, was found necessary.

For the experiments on the denervated muscle of the frog, the sciatic nerves were divided aseptically under ether anæsthesia, and the animals were kept at laboratory temperature (20° C.) for some 3 weeks, to allow degeneration to proceed to completion.

Results

Denervated mammalian muscle

The close arterial injection of doses between 0·001 and 0·1γ acetylcholine into the gastrocnemius of the cat, a week or more after section of the motor nerve, evokes a relatively quick, twitch-like contraction, which differs from the response produced by much larger doses in normal muscle, in the greater slowness of the relaxation (Fig. 1 A). As the dose is

increased the "quick" response develops a higher tension and relaxation becomes more rapid, but is interrupted by a secondary, slowly rising increase in tension (Fig. 1B). Still higher concentrations give responses in which the "slow" component of the response develops a greater tension than the "quick" phase (Fig. 1C) and, with doses producing approximately the maximum response, the diphasic character may only be revealed as a small change in the velocity of the rise of tension, the whole response giving a very nearly smooth curve (Fig. 5A). Although the method of close arterial injection is best adapted to the display of this

Fig. 1. A, B and C, decerebrate cat, contractions of denervated (12 days) gastrocnemius in response to 0·25γ, 1·0γ and 2·5γ Ach. by close arterial injection. D, spinal cat, contraction of denervated gastrocnemius in response to 20γ Ach. by "distant" injection into inferior mesenteric artery.

diphasic character of the response, it has frequently been recorded, as pointed out by Brown *et al.* with favourable preparations, in the responses to injections made into a more distant artery, such as the inferior mesenteric, and without any precaution to restrict the circulation to the gastrocnemius. No detailed study has been made of the effect of the duration of denervation upon the type of the response, but it appears that the slow component of the response and the sensitization to acetylcholine develop *pari passu*. In one experiment, in which four days only had elapsed after nerve section, the threshold dose was not lower than one-half of that for normal muscle, and the slow phase was only just visible. Six days after denervation, the high sensitivity and the slow phase of the response appeared both to be fully developed.

Electrical phenomena. Mammalian muscle, when deprived by degeneration of its motor nerve supply, is in a state of constant fibrillary activity. This fibrillation, which was examined in detail by Langley & Kato [1915*a*, *b*], is seen at its best when the exposed surface of the muscle is illuminated obliquely. In the present experiments, the cats used had had their sciatic nerves divided between 4 and 27 days previous to the experiment. The earliest day at which the fibrillation was seen was the sixth, the twitches being then infrequent and difficult to detect. In a cat of which the muscles were examined 4 days after nerve section, fibrillation was not seen. These observations are in agreement with those of Langley and Kato, who stated that fibrillation does not commence until the fifth day after nerve section. When the muscle is examined for electrical activity with concentric needle electrodes, it is found that the fibrillation is accompanied by an irregular series of diphasic action potentials (Pl. I, fig. 2*a*, *b* and *c*). It is difficult from records to determine the frequency of the irregularly discharging fibres, but, if the amplified potentials are led into a loud-speaker, the character of the discharge can be followed fairly closely by ear. The insertion of the needle excites some fibres, and rapid discharges of some 30 per sec. may be heard. This frequency rapidly falls and the discharging fibre appears to settle down to a regular rhythm at a low frequency. Measurements of a number of records show regularly recurring spikes at frequencies varying between 2 and 7 per sec. It is of interest to note that Langley and Kato, from direct inspection of the muscle, concluded that contraction in any one spot had a perfectly regular rhythm. The highest voltage recorded for these spontaneous action potentials was in the region of 30μV. This figure is close to that recorded from single muscle fibres excited by acetylcholine (25μV.) [Brown, 1937*b*] and leads to the conclusion that these spontaneous potentials are to be attributed to the activity of single muscle fibres.

The quick response of the denervated muscle to a small dose of acetylcholine is accompanied by an outburst of electrical activity greatly in excess of any previous spontaneous activity (Pl. I, fig. 2*a*). In general appearance the action potentials are very like those occurring in normal muscle, but the total duration of the discharge is, as might be expected from the maintained tension, very much longer. For instance, in a normal muscle, a few fibres may still be in activity one second after the injection of a dose producing a tension similar to that of a maximal nerve twitch, whereas a dose of acetylcholine of 1/100th the magnitude may, in a denervated muscle, produce a discharge lasting 5–6 sec. On the cessation

of the discharge evoked by such a small injection of acetylcholine, the spontaneous action currents reappear and continue unchanged.

Such is the picture of the electrical activity when the tension, produced by the injection of a small dose, falls steadily from a rapidly attained summit. As soon, however, as the dose becomes large enough to evoke any trace of sustained contraction, following the initial crest, the onset of this sustained effect is accompanied by a rapid diminution of the action potentials, which become small and infrequent in relation to the tension developed (Pl. I, fig. 2*b*). The reason for this suppression of action potentials is made evident if a concentration sufficiently high is given to cause a secondary slow response. In these circumstances, the quick phase is associated with an outburst of action potentials of considerable magnitude, which stops abruptly at, or close to, the commencement of the slow phase, during which no rapid action potentials whatsoever, due either to spontaneous activity or to the persisting effect of acetylcholine, can be detected (Pl. I, fig. 2*c*). The phenomenon is very striking if the amplified potentials are led to a loud-speaker; the spontaneous activity produces an irregular background of noise; the injection of acetylcholine is followed by an immediate roar which stops with extraordinary suddenness, to be followed by a complete silence, during which the muscle tension rises in the slow phase of the response. We have seen that the muscle, after a simple quick response to a small injection, returns immediately to a state of spontaneous fibrillation. After a larger injection, on the other hand, causing a secondary, slow response, the electrical "silence" accompanying the latter may persist long after the return of the muscle to its resting tension. The "silence" may persist, indeed, for as long as 10 min. after a single injection.

The duration of the silence appears to be directly proportional to the magnitude of the slow phase. In certain experiments, in which doses of such a concentration were used as to produce a slow phase of about $\frac{1}{4}$–$\frac{1}{2}$ of the tension of the quick phase, the myographic response became triphasic, the usual quick and slow responses being followed by a slow, third, development of tension. Electrical records from such a muscle revealed that the third contraction was accompanied by a renewed outburst of action potentials, which had been temporarily suppressed by the interpolated slow phase. There can be no doubt that this renewed outburst was due to acetylcholine lingering in the tissue spaces, or blood vessels, of the muscle. It is frequently observed that the re-opening of the artery to a muscle, closed temporarily for a retrograde injection of acetylcholine, is accompanied by a small response of the muscle as the

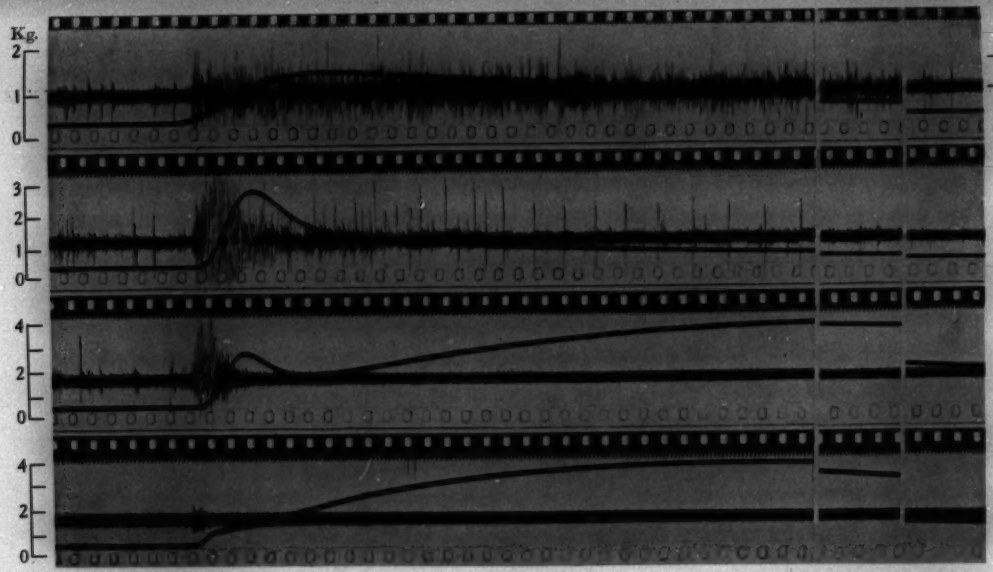

Fig. 2. Cat. Decerebrated. Isometric myogram of gastrocnemius 11 days after nerve section. Action potentials with concentric needle electrodes. Effect of close arterial injection of (*a*) 0·05γ Ach., (*b*) 0·5γ Ach., (*c*) 2·5γ Ach., (*d*) 2·5γ Ach. 1 min. after (*c*). All records except (*d*) show the spontaneous discharge preceding the injection. In all four records, the extra sections were taken 3·5 and 5·5 sec. after the injection. Time record, 10 msec.

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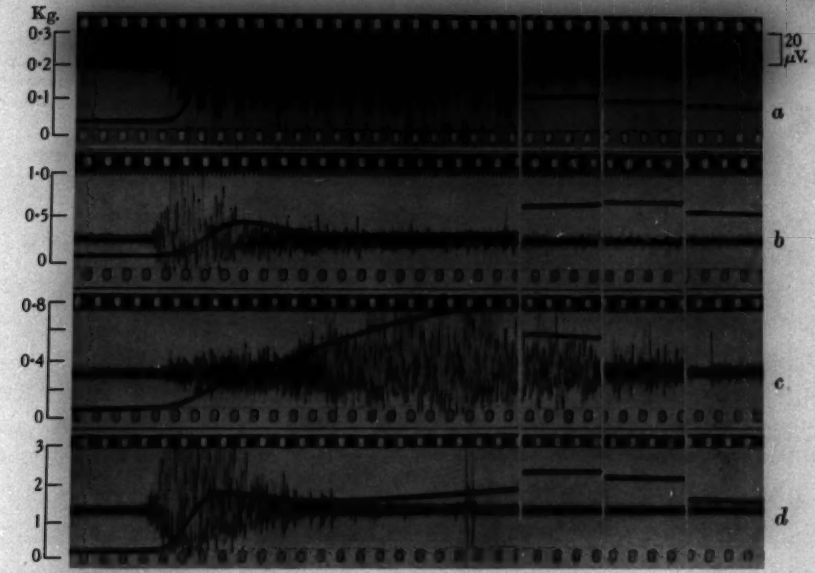

Fig. 6. Frog. Isometric myogram of gastrocnemius and action potentials recorded with belly-tendon leads. *a*, normal muscle, arterial injection of 1γ Ach. *b*, normal muscle, injection of 10γ Ach. *c*, denervated muscle (21 days), injection of 0·1γ Ach. *d*, denervated muscle, injection of 1γ Ach. In all four records the extra sections were taken 3 sec., 5 sec. and 7 sec. after the injection. Time record, 10 msec.

remaining acetylcholine is washed through it. Such a response is accompanied by a small outburst of action potentials.

Effect of acetylcholine injection on the response of denervated muscle to electrical stimulation. Gasser & Dale [1926] drew attention to the fact that the response of denervated muscle to direct electrical excitation was depressed, and even completely abolished, during and after an injection of acetylcholine of sufficient size to produce a contracture. They further observed that this depression did not involve to nearly so great a degree the reactivity of the muscle to a further injection of acetylcholine, which evoked a response of considerable magnitude when the muscle was still completely unresponsive to direct electrical excitation. This phenomenon has been examined in greater detail in relation to the two phases of the reaction observed with close arterial injection. It has been found that concentrations of acetylcholine, sufficient only to evoke the quick phase, do not materially depress the response to direct excitation, except in so far as the stimulus falls among fibres, many of which must be refractory (Fig. 3A).

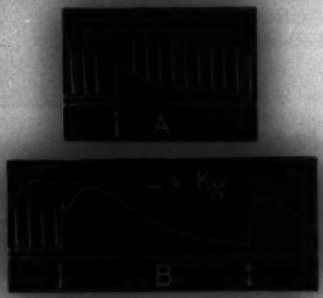

Fig. 3. Cat. Chloralose. Contractions of gastrocnemius, 18 days after nerve section, maximal direct induction shocks every 1·5 sec. A, close arterial injection of 0·1γ Ach. during intermission of two shocks. B, successive injections of 1γ Ach. with no intermission of stimulus.

If a dose large enough to display the slow component is given, the depression of the effect of electrical excitation becomes very evident (Fig. 3B). It is to be noted that the maximum depression is not coincident with the onset of the slow phase, not being obtained until its crest is reached; if reference is made to Pl. I, fig. 2*c*, it is seen that, similarly, complete suppression of action potentials in the dual response does not occur until the contracture is nearly maximal. A further injection of acetylcholine, made during the period of depressed electrical excitability, shows that the depression also involves the response to acetylcholine, but that only the quick component is seriously affected (Fig. 3B). This depression is very clearly demonstrable if the action potentials are recorded (Pl. I, fig. 2*c* and *d*). The first injection produces the typical outburst of diphasic potential changes, followed by the "silence". A second injection, made one minute after the first, and during the persisting silence, evokes almost exclusively the slow

component, oscillatory action potentials being restricted to a very small initial outburst.

It seems clear, then, that the depression produced by the slow phase of a first response involves (1) the spontaneous fibrillar activity; (2) the electrical excitability; and (3) the quick phase, but not the slow phase, of a further response to acetylcholine.

Action of eserine. Brown *et al.* [1936] showed that eserine produced only a small potentiation of the effects of acetylcholine injected arterially into normal muscles. In so far as the quick phase of the response is

Fig. 4. Cat. Decerebrated. Contractions of gastrocnemius, 8 days after nerve section, in response to 3 close arterial injections of 0·5γ Ach. Between A and B arterial injection of 80γ eserine. C, 30 min. after B.

Fig. 5. Cat. Decerebrated. Contractions of gastrocnemius, 20 days after nerve section, in response to close arterial injections of A, 2·5γ Ach., B, 50γ carbaminoylcholine and C, 21 min. later, 10γ Ach.

concerned, this applies also to denervated muscle. Little or no change was observed in the magnitude of the tension of the quick phase of the response to acetylcholine, after injections of eserine, in concentrations even higher than those necessary to produce, in a normal muscle, a full potentiation of the response to nerve volleys. The slow phase of the response shows, however, an increase both in the tension developed and in its duration (Fig. 4).

In connexion with the actions of eserine, the effects on the muscle of a stable choline ester are of interest. Injections of the unstable choline esters made into normal muscles, produce a response which has no after effects; but if the destruction of these esters is prevented, the stimulating

effect is followed by a long-lasting depression [Brown *et al.* 1936]. On normal muscle carbaminoylcholine has a small stimulating effect, followed by a progressively increasing and apparently irreversible depression of the response of the muscle to nerve stimulation [Bacq & Brown, 1937]. Fig. 5 shows the effect of carbaminoylcholine on denervated muscle. At A, 2·5γ acetylcholine were injected, and this was followed after 15 min. by an injection of 50γ carbaminoylcholine. The quick phase of the response is very like that following the much smaller dose of acetylcholine, but the slow phase is much more prolonged. The depression associated with the slow phase long outlasted the obvious mechanical manifestation; 21 min. after the injection of carbaminoylcholine the muscle was still unresponsive to direct electrical excitation, and a further injection of acetylcholine then produced only a reduced response, which appeared to possess no quick component.

Curarine. Brown *et al.* [1936] reported that both components of the response of denervated muscle to acetylcholine were affected by curarine. This finding has been confirmed, using very small doses of curarine administered by arterial injection. The quick and the slow phases of the response are equally depressed by curarine thus administered.

Adrenaline. The depressant effect of adrenaline on the response of denervated muscle to acetylcholine was investigated in some detail by Gasser & Dale and further observations were made by Dale & Gaddum [1930]. In the course of the experiments on normal mammalian muscle, it was found that the response of the muscle to acetylcholine was not significantly affected by adrenaline, in doses which the previous investigations had shown to be sufficient completely to annul the response of the denervated muscle. This led to the expectation that, by suitable dosage of adrenaline, it might be possible to dissociate the two phases of the response, and to allow one to persist in the absence of the other. An animal was therefore prepared for close arterial injections *via* the tibial artery, and for distant arterial injections into the aorta *via* the inferior mesenteric artery, the method used by Gasser & Dale. It was found that the muscular response to the distant arterial injection was completely absent, if the injection was made during the pressor effect of 0·5 mg. adrenaline, whereas there was no significant alteration in the response of the muscles to the close arterial administration of a much smaller dose of acetylcholine.

Normal muscle of frog

The injection into the sciatic artery supplying the gastrocnemius of the frog, of 0·1 c.c. of Ringer's solution containing between 0·1 and 1·0γ of acetylcholine has a very striking effect. The muscle abruptly contracts, and over its whole surface is seen a diffuse, irregular flickering and indentation, which is most evident beneath the glistening aponeurosis of the distal end. Mechanical records show the rapid attainment of a tension which, with the larger doses, may be greater than that of the maximal twitch. In some experiments, with fresh muscles, tensions in excess of 200 g. were recorded with injections of 1·0γ of acetylcholine.

In the majority of the experiments, the lowest dose of acetylcholine which was found to give a recordable shortening of the muscle was 0·05γ in 0·1 c.c. In one of the experiments, in which the injections were made into the tibial artery with the muscle under natural circulation, a dose of 0·01γ produced a contraction, 1γ of eserine having been previously injected into the muscle. The initial rise of tension is followed by a short plateau and a slow relaxation (Pl. II, fig. 6 and Fig. 7). With larger doses (1–10γ), the relaxation is interrupted by a second, much slower development of tension, and the record shows something approaching the diphasic response seen in denervated mammalian muscle (Pl. II, fig. 6, Figs. 7 and 8). The distinction between the two phases is, however, never so clear as in the mammal, and the slow component of the response seldom shows the development of a much greater tension than the quick phase.

Electrical phenomena. The quick phase of the response in the normal muscle of the frog, as in the denervated muscle of the mammal, is accompanied by an outburst of asynchronous action potentials (Pl. II, fig. 6*a*). These are easily recordable by belly-tendon leads and potentials as high as 50μV. may be observed. With doses up to 1γ the discharge starts slightly before the mechanical change, and rapidly increases and remains at a considerable amplitude for some 6 sec., after which it slowly declines and ceases. The total duration of the response may be more than 40 sec. In this respect, the reaction of the normal frog muscle resembles that of the denervated mammalian muscle much more closely than it resembles the relatively evanescent responses of the mammalian muscle with intact innervation.

With increase in the concentration of acetylcholine injected, there is not a comparable increase in the amplitude and duration of the oscillatory action potentials. For instance, in the experiment illustrated in Pl. II, fig. 6*b*, the injection of 10γ acetylcholine produced a big initial outburst,

followed by a quick fall to a low level of activity. This almost complete cessation of oscillatory action potentials is coincident with the development of the slow component of the response. In this respect there is again a close similarity between the frog's normal muscle and the denervated mammalian muscle.

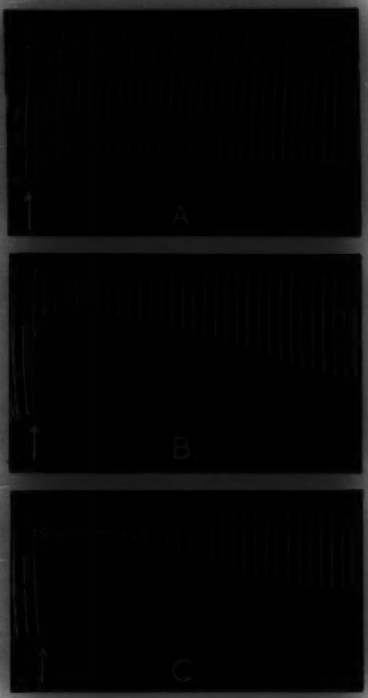

Fig. 7. Frog. Isotonic record of contractions of normal gastrocnemius in response to maximal nerve volleys every 2·5 sec. Injection into sciatic artery of A, 0·1γ Ach. B, 0·2γ Ach.; C, 1γ Ach.; with no remission of stimulus.

Effect of acetylcholine on the response of frog muscle to motor nerve volleys and to direct stimulation. In general, the effect of acetylcholine on the direct excitability of frog's muscle is again similar to that which it produces on the denervated muscle of the mammal, the quick component of the response having little effect, whereas the slow component is associated with depression, which, however, is never so complete as in the case of the denervated mammalian muscle. The presence in the frog of the normal innervation introduces additional factors of interest.

With doses of acetylcholine of less than 1γ, there is a little depression of the response to direct or indirect excitation of the muscle (Fig. 7). Amounts of the order of 1γ, although producing a large and prolonged outburst of action potentials, are nevertheless associated with an obvious depression of the response to nerve stimulation. With the greater doses, 5–10γ, in the responses to which the slow component is obvious, there is a more or less complete suppression of the response to a nerve volley, and this depression also affects, though to a lesser degree, the response to

direct excitation of the muscle (Fig. 8*a* and *c*). In normal mammalian muscle, the tetanic response to acetylcholine is not associated with any depression of the motor nerve twitch, even when this is set up during the acetylcholine response; and similarly, the quick phase of the response in denervated mammalian muscle does not depress the excitability of the muscle to direct stimulation. The constant association of the depression of excitability with the slow component appears to justify the use of the appearance of depression as a criterion of the presence of the slow component. In frog muscle it is frequently difficult, in the absence of a simultaneous record of action potentials, to decide whether a response is wholly of the quick type associated with oscillatory action potentials; but if the response is accompanied by any considerable depression of excitability, it is probably legitimate to conclude that some at least of the tension developed is attributable to the slow component. If this deduction is accepted, it becomes doubtful whether the slow component is ever entirely absent from the responses of the frog's gastrocnemius to acetylcholine, since, even when the injection is of such a concentration as to be associated with prolonged and ample action potentials, there is some depression of the response to intrapolated nerve volleys.

Effects of atropine. High concentrations of atropine injected arterially into the frog's gastrocnemius suppress, more or less completely, the response of the muscle to stimulation of its motor nerve. The amount required to produce the effect is 100γ of atropine sulphate, injected in a volume of 0·1 c.c., i.e. 1 in 1000; with this dose, motor nerve stimulation rapidly becomes either totally ineffective, or the response is reduced to a very small proportion of its original tension. The effect is rapidly reversible and, with continued perfusion, complete recovery may ensue in some 30 min. This concentration of atropine depresses, but does not abolish the muscular response to acetylcholine. Both phases of the response are depressed, but there is some evidence of a differential sensitivity of the slow component. A typical experiment is illustrated in Fig. 8. Into the gastrocnemius of a frog, stimulated through its nerve at intervals of 2·5 sec., was injected 10γ of acetylcholine. The response showed clearly the depression associated with the slow phase. The muscle then received 100γ atropine arterially, and failure of the response to nerve stimulation followed. During direct stimulation of the muscle and 5 min. after the atropine, 10γ of acetylcholine were again injected. The resulting contraction is much smaller than that produced by the same dose before atropine, and it will be seen, further, that there is very little depression of the effects of muscle stimulation. After 35 min. the effects of the atropine

had passed off, and the response to injected acetylcholine had returned to its previous appearance.

Electrical records in similar experiments have shown that the reduced response after atropine is associated with an outburst of action potentials, not significantly different from that accompanying the response before atropine.

Fig. 8. Frog. Isotonic record of contractions of normal gastrocnemius. A, maximal shocks to nerve every 2·5 sec. Injection of 10γ Ach. Three contractions marked (·), direct stimulation of muscle. Between A and B arterial injection of 100γ atropine. B, direct stimulation of muscle, injection of 10γ Ach., at the points marked (·) shocks to nerve. C, 35 min. later. Maximal shocks to nerve, injection of 10γ Ach. Four contractions marked (·), direct stimulation of muscle.

Effects of curarine. Both phases of the response to acetylcholine are depressed by curarine. No clear evidence of a differential sensitivity was obtained, but the slow phase appears a little more resistant. The effects of curarine are illustrated in Fig. 9. The arterial injection of 2γ curarine between A and B reduced the response to nerve stimulation, and apparently reduced equally the two phases of the acetylcholine effect. Further injections of curarine, between B and C greatly depressed the responsiveness to acetylcholine, without completely blocking neuromuscular conduction. In other experiments it was found that the effects of 10γ of acetylcholine, which is the highest concentration yet given, could be almost completely abolished by the previous administration of 10γ curarine. Langley [1914] found that curarine completely suppressed the effects of nicotine applied locally to the region of the nerve ending, but did not appreciably antagonize the effect of stronger nicotine on the non-neural portion of the muscle. It is probable that I did not test the effects of acetylcholine in concentrations equivalent to those of nicotine, applied by Langley to the non-neural muscle end. If I had done so, it seems likely that the paralysing effect of curarine might have been overcome.

Effect of eserine. Amounts of eserine from 1γ up to 100γ in 0·1 c.c. were administered to the frog's gastrocnemius by arterial injection. The smaller doses of eserine were without obvious effect on the response of the muscle to nerve volleys, delivered at frequencies varying between 1 per 2 sec. and 1 per min. The response of the muscle to acetylcholine was also little affected, with the exception of a possible slight increase in the slow

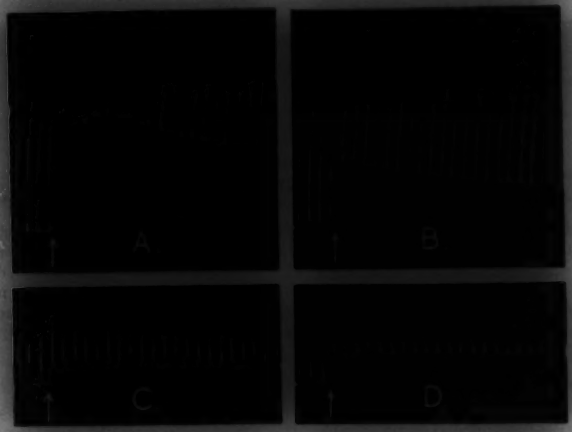

Fig. 9. Frog. Isotonic record of contractions of normal gastrocnemius, maximal shocks to nerve every 2·5 sec. Arterial injections of A, 1γ Ach. Between A and B, 2γ curarine, B, 1γ Ach. Between B and C, 8γ curarine. C, 1γ Ach. D, 2·5γ Ach. 10 min. later.

component, as judged by the degree of depression of the effects of nerve stimulation. With larger doses, the response of the muscle to nerve stimulation and to acetylcholine was depressed.

This relative inactivity of eserine on frog's muscle made it of interest to test the effect on the muscle of the stable choline esters. The threshold dose of carbaminoylcholine is of the order of 1γ in 0·1 c.c. An injection of 20γ produced a contraction similar to that following 1γ acetylcholine and associated with very obvious fibrillary twitchings of the muscle. The response was easily distinguishable from that of the otherwise equivalent dose of acetylcholine by the considerable depression of the response of the muscle to stimulation, both through the nerve, and directly. This depression produced by carbaminoylcholine does not, however, proceed to complete paralysis of the muscle, as it does in the normal and denervated

mammalian muscle, but gradually passes off. In the particular experiment described, the excitability of frog's gastrocnemius had returned approximately to normal some 25 min. after the injection. With smaller doses of carbaminoylcholine (5γ), the depression is equally severe, but recovery takes place sooner.

Denervated muscle of frog

In three frogs, the sciatic nerve was cut and allowed to degenerate for three weeks. The muscles were prepared for injection in the usual way. No spontaneous fibrillation was detected. The threshold dose of acetylcholine for such a muscle is about 10 times lower than for the normal

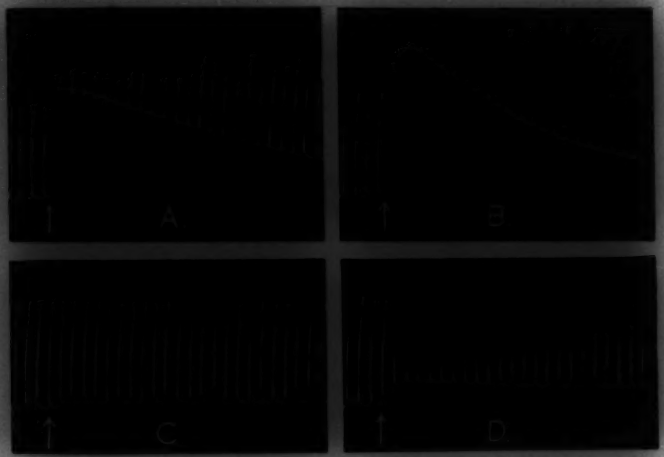

Fig. 10. Frog. Isotonic record of contractions of gastrocnemius 22 days after nerve section. Maximal direct stimuli every 2·5 sec. Arterial injections of A, 0·1γ Ach.; B, 0·5γ Ach. Between B and C 10γ curarine. C, 0·5γ Ach.; D, 5γ Ach.

muscle. Just recordable contractions were obtained with injections of 0·005γ. With doses of 0·1γ, contractions were obtained comparable to those occurring in normal muscle with 1γ (Pl. II, fig. 6 and Fig. 10). In general characters, apart from the difference in threshold, there is little difference between the response of the normal and the denervated muscle. The injection of 0·1γ produces the quick contraction accompanied by a prolonged outburst of diphasic action potentials (Pl. II, fig. 6*c*). With 1γ, as in

the normal muscle with 10γ, the initial quick response is followed by the slow phase, during which action potentials are suppressed (Pl. II, fig. 6*d*).

The most obvious difference from the normal muscle is the extent and duration of the depression of the response to direct stimulation, produced by the injection of a dose large enough to evoke the slow component (Fig. 10B). In this respect the denervated muscle of the frog resembles closely the denervated mammalian muscle. In the particular experiment illustrated in Fig. 10, complete recovery did not take place until 32 min. after the injection. In Fig. 10 is also shown the action of curarine upon the denervated muscle. Between B and C 10γ of curarine was injected and in C 0·5γ of acetylcholine was given as in B. The response is reduced almost to the vanishing point. In D, 5·0γ acetylcholine were injected and a small response was observed, together with severe depression of the effect of direct stimulation. Observation of the muscle during this response showed that there was complete absence of the visible fibrillation, which is a normal accompaniment of such an injection. Presumably, the response showed only the slow phase.

Discussion

In a previous paper [Brown, 1937*b*], it was shown that the response of normal mammalian muscle to the close arterial injection of acetylcholine is accompanied by an outburst of asynchronous action potentials. Similar potentials have been shown, in the present investigation, to accompany the injection of appropriate concentrations of acetylcholine into the arteries supplying the denervated muscle of the mammal and the frog, and also the normal frog muscle. The possibility of artefacts appears to be adequately excluded by the observation that a control injection of saline is without effect, and that, under certain conditions, an injection of acetylcholine may evoke a big mechanical response (contracture), which is unaccompanied by any appreciable electrical activity (*vide* Pl. I, fig. 2*d*). The interpretation which Brown *et al.* gave to the excitation of normal muscle by acetylcholine, namely, that propagated contractions were set up in the region of the end plate, receives further confirmation from these experiments. The fact that the response of a muscle, after degeneration of its motor innervation has a similar, rapid phase, excludes the possibility that the response previously described might have been due to the excitation by acetylcholine of the terminal portions of the motor axon.

Certain differences obviously exist between the quick phase of the response of denervated mammalian muscle and the response of normal

muscle to acetylcholine, the most striking being the smallness of the dose required to excite the denervated muscle and the long duration of both the mechanical response and the accompanying action potentials. It has been found in another tissue, the superior cervical ganglion, in which acetylcholine is involved in the synaptic transmission process, that decentralization by degenerative section of the preganglionic fibres brings about a disappearance of most of the acetylcholine available for extraction [Brown & Feldberg, 1936]. The probable association of a high acetylcholine content and a high activity in cholinesterase suggested that preganglionic section might reduce the activity of the esterase present in the ganglion. Experiments which Dr F. v. Brücke has just completed in this laboratory confirm this. In previous papers, evidence has been presented of the existence, in normal muscle, of a high concentration of esterase at the end plates and, at first sight, a reduction in this esterase might be taken as offering an easy explanation of the sensitization of muscle by denervation. An inhibition by eserine of the esterase of normal muscle can be produced, sufficiently profound to cause the persistence of the effects of a single nerve volley for more than a second; yet eserine has relatively little effect on the sensitivity of normal muscle to injected acetylcholine. It seems, therefore, that the sensitization produced by denervation cannot be due, in its entirety, to disappearance of esterase, but that some more fundamental change must take place.

The prolonged discharge observed in denervated muscle is probably a function only of the low threshold of the muscle to acetylcholine. There is reason to believe that acetylcholine injected into a muscle, from which the blood has been drained, can persist in the vascular spaces for a considerable time, and some preliminary experiments show that normal and denervated muscles do not differ in this respect. In a previous paper [Brown, 1937*b*], the suggestion was made that the continued discharge of a single muscle fibre could best be accounted for on the assumption of a maintenance at the end plate of a stimulating concentration of acetylcholine, by renewal from what lingers in the tissue spaces of the muscle; the constant destruction of the acetylcholine reaching the end plate, by the cholinesterase concentrated at the nerve ending, necessitating such constant renewal for the maintenance of a stimulating concentration. The same considerations would explain the dependence of the response of the normal muscle to injected acetylcholine on the rapidity with which the injection is made. The response of the denervated muscle to injection of acetylcholine is not only elicited by a much smaller dose, but is much less dependent on the speed of injection. The response of denervated muscle,

indeed, came early to notice for the reason that it could be elicited by distant arterial, and even by intravenous, injection. It is highly probable that part, at least, of this change of reaction with denervation is due to disappearance of the concentration of esterase associated with the nerve endings.

On the grounds, therefore, of low threshold and of relative independence of rapid access, it is easy to account for the prolonged character of the outburst of oscillatory potentials. With higher concentrations of acetylcholine, the quick response is replaced and suppressed by the slow response.

The slow phase of the response is not accompanied by oscillatory action potentials; it is clearly of the nature of a non-propagated contraction of the fibres; in other words, a contracture proper. The contracture is accompanied by three phenomena: (1) suppression of the spontaneous action potentials; (2) suppression of the initial, quick phase of the response of the muscle to a further injection of acetylcholine, and of the action potentials which accompany this quick phase; and (3) depression of the excitability of the muscle to directly applied electrical stimuli. In other words, it appears that the existence in the muscle of a contracture so alters the conducting mechanism of the fibre, that the excitation wave set up, either at the end plate or elsewhere, can no longer be propagated. Such a muscle is still excitable by acetylcholine, but the response lacks the component of propagated disturbances, and is merely a renewed contracture. This phenomenon explains the anomaly to which Gasser & Dale [1926] refer, namely, that a denervated mammalian muscle, after responding to a large dose of acetylcholine, may be completely inexcitable electrically, but still responsive to a further injection of acetylcholine. One remarkable feature of the non-propagated contracture is that its effects may long outlast the development of tension in the muscle. After a series of contracture-producing doses of acetylcholine, the spontaneous activity of the muscle may be absent for long periods and may, indeed, never reappear during the experiment; and the same applies to the quick phase of the response to further injections of acetylcholine. Once a large contracture has been evoked, the quick component becomes increasingly small in the responses to successive further injections, the threshold of the muscle for acetylcholine rises, and the contractions produced by maximal shocks applied to the muscle are permanently reduced in tension. It appears that, when once a contracture of any magnitude has been evoked, it leaves behind some, more or less permanent, derangement of the conducting system of the muscle fibre.

From the experiments on denervated mammalian muscle, no information can be obtained of the locus of the excitatory actions of acetylcholine, although the presumption must be that the endplate is the excited region. More information on this point can be obtained from the experiments on the frog.

In general, the reactions to acetylcholine of the normal innervated frog's muscle closely resemble those of mammalian muscle after denervation; but the threshold is much higher, and approximates more closely to that of normal mammalian muscle. The existence of oscillatory action potentials in connexion with the response of frog's muscle to acetylcholine has been the subject of some discussion; but in all previous experiments, in which the substance was injected into the arterial tree of the muscle, oscillatory potentials have been observed in the initial phases of the response [cf. v. Neergaard, 1924; and Schäffer & Licht, 1926*a*]. v. Neergaard, indeed, describes the appearance of fibrillary twitchings in the muscle. Such effects, however, evoked by distant arterial injection, must have been weak and inconstant, and were, in fact, dismissed by those who observed them as insignificant and accidental. The injection of acetylcholine at close range into frog's muscle shows, however, that the outburst of fibrillary twitchings is as constant and significant a feature of the response of the muscle as the more generally known contracture. This finding that acetylcholine, appropriately injected, sets up propagated contractions in frog's muscle, removes a former difficulty in regarding it as the neuro-muscular transmitter in the frog, as in the mammal.

The contracture, which occurs in frog muscle with high concentrations of acetylcholine, like the contracture of denervated mammalian muscle, depresses the excitability of the muscle to directly applied electrical stimuli, but has, in addition, the power of depressing to a much greater extent the response of the muscle to stimuli applied through the nerve. There can be little doubt that the propagated contractions are set up only in the region of the nerve ending, since the action of acetylcholine belongs to the nicotine-like group of its actions and, as Langley [1907] has shown, nicotine sets up fibrillary twitchings only in the neural region. Not only is the neural region of the muscle the region where twitching is produced, but, according to Langley [1907], the "tonic" contraction is evoked more readily here than elsewhere in the muscle. These facts, together with the observation of the early blocking of the response to nerve volleys, seem to point with reasonable certainty to the region of the nerve ending as the starting point of the contracture.

The experiments of Sommerkamp [1928] suggested that only certain regions of the frog's gastrocnemius could give the contracture, and Langley's [1907–14] observations with nicotine, and those of Wachholder & Ledebur [1930] with acetylcholine, showed clearly the varying characters of different muscles in this respect. The present experiments indicate that in denervated mammalian and frog's muscle (gastrocnemius) the contracture eventually involves all the fibres of the muscle, probably throughout their length, since there is usually a complete and long lasting suppression of direct excitability, if a sufficiently large dose of acetylcholine is injected. With frog's normal muscle, the frequent complete abolition of indirect excitability and the persistence, much reduced, of direct excitability would suggest that all the fibres are involved, but that the contracture does not extend to the full length of the muscle fibre. Variations in the threshold of different fibres may well exist, but the methods of recording used were not such as to reveal them.

Antagonistic drugs, so far as these have been tested, show little discrimination between the two phases of the response to acetylcholine. Curarine, in the denervated mammalian muscle, equally depresses both phases, and in the frog's muscle no clear evidence of differential susceptibility of the two phases could be found. This agrees, in general, with Langley's [1907] observations on the antagonism between nicotine and curare, when acting on the neural region of frog's muscle.

The only substance providing some evidence of a differential paralysis of the two phases is atropine, which appears to affect the contracture more readily than the tetanic component—a finding in accordance with that of Feng [1936] on the neuromuscular contracture of the toad, and that of Dale and Gaddum [1930] on the contracture of the isolated, denervated diaphragm of the cat. The effect of atropine on the normal frog's muscle, in producing neuromuscular block and leaving a large part, at least, of the acetylcholine response, is anomalous. Other substances characteristically producing neuromuscular block, such as curarine, have been found to suppress the responsiveness of the muscle to acetylcholine before the nerve effect was seriously involved [Brown *et al.* 1936]. It must be remembered that very high concentrations of atropine are necessary ($1 : 10^2$), and that the effect is relatively transient. A possible explanation of such action, compatible with chemical transmission, is that atropine so administered prevents or depresses the liberation of acetylcholine at the nerve endings.

The absence of any antagonistic action by adrenaline, when acetylcholine is injected at close range into denervated mammalian muscle,

contrasts strikingly with complete elimination of the response, when a more distant injection is made into the circulating blood. Dale & Gaddum [1930] suggested that adrenaline acted by producing a reduction of the permeability of the capillary walls. The present findings are not incompatible with this view, if the fact is taken into consideration that by the close arterial injection, the acetylcholine is forced, under considerable pressure, into arteries which have been emptied of blood.

The observations made on the action of eserine throw some light on the origin of the contracture phase of the acetylcholine response. That eserine had little effect on the quick phase was to be expected, from the relatively small effects it produces on the response of normal mammalian muscle to injected acetylcholine; but the prolongation of the contracture by eserine would suggest that some cholinesterase, at least, persists in the muscle fibre after denervation. In the frog, eserine has been found to have little action other than depression of the response to nerve stimulation, there being no sign of the typical repetitive discharge of the muscle in response to single nerve volleys, such as is seen in the mammal. Feng [1936] has described occasional irregular "supramaximal" twitches as occurring under eserine in the toad, and Feng & Shen [1937] have recently shown that toad's and frog's muscle, under eserine, develop a contracture at the neural region, in response to suitably timed repetitive stimuli. This contracture bears many resemblances to the acetylcholine contracture described in this paper, and in particular, as Feng & Shen point out, it is invariably associated with a considerable degree of neuromuscular block. This phenomenon of contracture in frog's muscle, when acetylcholine persists in it, and the accompanying neuromuscular and conduction block, must obviously complicate the effect of eserine on the response of such a muscle to a nerve volley.

Although the most striking action of eserine on normal mammalian muscle is the initiation of repetitive discharge in response to single nerve volleys, an equally important effect is the depression of the response to nerve stimulation which results either from an injection of acetylcholine [Brown *et al.* 1936], or from tetanic stimulation of the nerve above a very low limiting frequency [Bacq & Brown, 1937]. This effect of eserine can be attributed to its prevention of the destruction of acetylcholine, whether liberated at the nerve ending or introduced artificially. In denervated muscle and normal frog muscle, injected acetylcholine can reach a sufficient concentration at the end plate, even in the absence of eserine, to produce that depression of conduction in the muscle fibre which is associated with the non-propagated contracture. It must be

left to further experiment to explore the connexion, if any, between this phenomenon and the depression by acetylcholine of the response of the normal muscle, under eserine, to nerve stimulation.

The evidence presented in this paper gives further support to the view that neuro-muscular transmission, in the amphibian, as well as in mammalian muscle, is effected by liberation of acetylcholine at the nerve endings. The facts presented give incidental support to the suggestion of a concentration of cholinesterase at the mammalian motor nerve endings, removed by denervation; and the fact that the responses of the frog's gastrocnemius to acetylcholine resemble those of the denervated, rather than the normal, mammalian muscle, suggests that there is no such high concentration of cholinesterase at the amphibian motor nerve endings.

Summary

1. Denervated mammalian muscle is in a state of constant spontaneous activity, which produces a continuous series of diphasic action potentials.

2. The close arterial injection of acetylcholine into such a muscle evokes a double mechanical response, consisting of a quick initial contraction, followed, if the concentration injected is sufficiently high, by a slow contracture.

3. The quick response to injected acetylcholine is accompanied by an outburst of action potentials, like that occurring in normal muscle, but persisting longer.

4. With larger doses, this initial outburst is cut short, with the onset of the slow contracture, during which no rapid action potentials can be detected. This "silence" may long outlast the development of tension in the muscle.

5. During the "silence" renewed injection of acetylcholine produces only the slow contracture, with no accompanying action potentials and the response of the muscle to direct electrical excitation is depressed.

6. Injection of acetylcholine directly into the arteries of the frog's normal gastrocnemius produces responses similar to those of a denervated mammalian muscle. Small doses of acetylcholine cause a quick contraction of high tension, associated with a long lasting outburst of rapid diphasic action potentials. Larger doses cause an initial quick response, followed by a contracture, which terminates the electrical outburst.

7. Denervation increases the excitability of frog's muscle to both phases of the acetylcholine action.

8. The contracture evoked by acetylcholine in frog's muscle depresses the indirect excitability and, to a smaller degree, the direct excitability of the muscle.

9. Reasons are given for assuming that acetylcholine excites propagated, "All or Nothing", responses at the region of the nerve ending in frog's muscle and, by analogy, in denervated mammalian muscle.

I wish to express my thanks to Sir Henry Dale for the very helpful interest he has taken in this investigation.

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THE DURATION OF THE RECOVERY PERIOD FOLLOWING STRENUOUS MUSCULAR EXERCISE, MEASURED TO A BASE LINE OF STEADY, MILD EXERCISE

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The recovery period after severe exercise of short duration has been studied by several groups of workers [Hill *et al.* 1924; Best *et al.* 1929; Gemmill, 1931; Solandt & Ridout, 1933]. All are agreed that the respiratory exchange does not return to the basal level of complete rest for at least 1½ hours after such exercise. Gemmill believes that recovery takes even longer than 1½ hours.

In all these experiments the "basal" state was used as the base line from which the duration of the recovery period was determined. There is no fundamental reason for using "basal" metabolism as the base line in experiments of this type. The "basal" state is only one of many possible conditions in which the factors influencing metabolism can be controlled. It is also a condition which is rarely encountered in actual life, and can only be attained after prolonged rest. Prof. A. V. Hill therefore suggested that recovery after strenuous exercise might be more rapid if a more natural base line, such as that provided by mild, continuous exercise, were used. This is certainly the case with the recovery heat production of nerve [Feng & Hill, 1933].

Apparatus and technique. All the determinations of respiratory metabolism were done by the modified Douglas bag technique previously described [Solandt & Ridout, 1933]. The mild exercise used as the base line in these experiments was performed on a simple bicycle ergometer fitted with a friction brake. The subject pedalled in time with an electric metronome and was able to attain a very constant rate of pedalling. The strenuous exercise used was standing running, as fast as possible, for 30 sec. This type of exercise was chosen because the duration of the recovery period to the "basal" state had previously been determined following such exercise.

Experiments. As in the work previously reported [Solandt & Ridout, 1933], two types of experiments were performed. (1) Control experiments during which the subject pedalled steadily, on the bicycle ergometer, for about 2 hours. After a preliminary period of 15 min. to allow for attainment of the steady state, nine collections, each lasting for 8 min. and separated by 3 min. intervals, were taken. (2) Exercise experiments. After a preliminary period of 15 min. pedalling, one 8 min. collection was taken. The subject then stepped off the ergometer and ran, in place, as fast as possible, for 30 sec. Resuming his seat on the bicycle he continued pedalling at the former rate. The first post-exercise collection was begun at the start of the exercise and continued for 10 min. Successive collections were then taken as in the control experiments, continuing for 1½ hours after the strenuous exercise.

Results

Duration of the recovery period. In the first series of experiments, the subject pedalled the unloaded ergometer at a rate of 97 rev. per min. For the first few experiments this exercise gave an oxygen consumption of about 400 c.c. per min. (The "basal" oxygen consumption of the same subject averaged 238 c.c. per min.) Recovery from the superimposed standing running appeared to be complete in about 40 min. With continued practice, however, the subject's oxygen consumption fell to about 350 c.c. per min. for the same rate of pedalling. Coincident with this fall in oxygen consumption there was an increase in the duration of the recovery period to 70–90 min. The carbon dioxide output and oxygen consumption curves for these experiments are so similar to those obtained with recovery to the basal state, in the same subject (T.B.), that they have not been reproduced. The usual period of carbon dioxide retention from 30 to 60 min. after the exercise is particularly prominent.

When the maximum lowering of oxygen consumption due to training had apparently been obtained, a second series of experiments was begun. In these the subject pedalled at a rate of 180 rev. per min. with a load of 500 g. on the belt of the ergometer. This exercise gave an oxygen consumption of about 660 c.c. per min. Four exercise and four control experiments were done at this level of oxygen consumption. The results are plotted in Fig. 1. The curves show the oxygen consumptions and the carbon dioxide outputs for the exercise experiments. As in the previous paper, the ranges of oxygen consumption and of carbon dioxide output during the steady pedalling are indicated, in the figure, by the average high and the average low values obtained in the control experiments. Recovery is considered to be complete when the oxygen consumption and

carbon dioxide output return within, and remain within, these control limits.

Fig. 1 shows that recovery, thus defined, is complete within 20 min. in Exps. 2 and 4, within 35 min. in Exp. 1 and within 45 min. in Exp. 3.

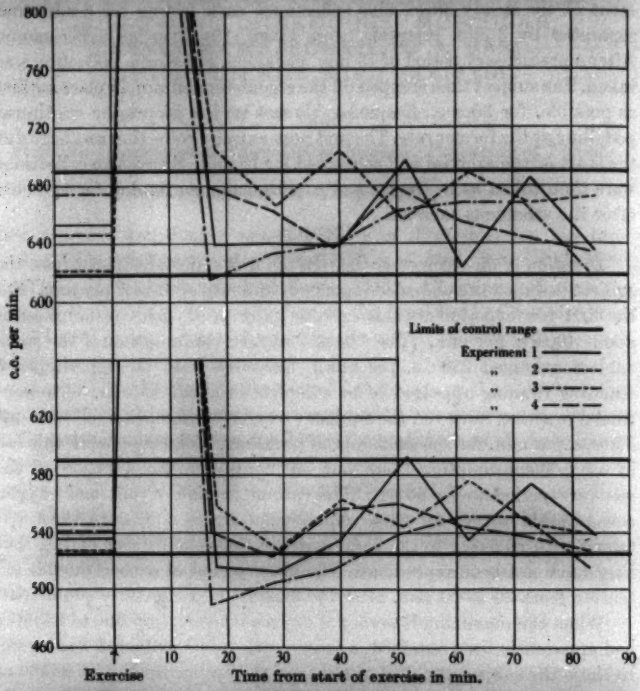

Fig. 1. Showing the oxygen consumption (upper graph) and carbon dioxide production (lower graph) following 30 sec. standing running at top speed. Recovery is to a steady state of mild exercise. The "limits of the control range" are the limits of metabolism during that steady state as determined in separate experiments.

It is also apparent that recovery may have occurred even sooner than this in Exps. 2 and 4 since the first collection after exercise is within normal limits. In Exps. 1 and 3 the post-exercise period of carbon dioxide retention is still apparent but seems to end earlier than when recovery is to the basal state.

In order to investigate the immediate post-exercise period in greater detail a further series of experiments was performed in which the post-exercise collections were made over much shorter intervals, and recovery was only followed for 35–40 min. The results of these experiments are shown in Fig. 2. In the four exercise experiments recovery is complete in

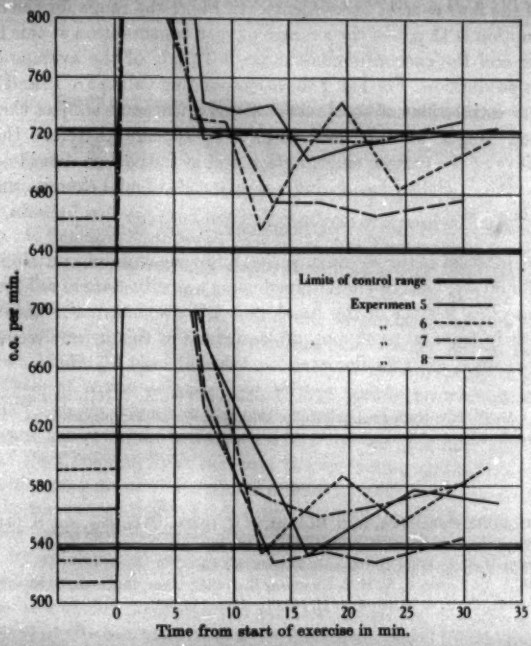

Fig. 2. Showing the oxygen consumption (upper graph) and carbon dioxide production (lower graph) following 30 sec. standing running at top speed. Similar to Fig. 1 but showing the early part of recovery in greater detail.

20–30 min. In these experiments there is a definite, post-exercise elevation of the oxygen consumption. This phenomenon has been discussed in the previous paper. Unfortunately the subject did not exercise quite as vigorously for Exps. 5–8 as for Exps. 1–4. The average excess oxygen for Exps. 1–4 was 4·3 l. and for Exps. 5–8 3·6 l.

Observations on the steady state. The control experiments in these series provide further data concerning the "steady state". The exercise per-

formed was well within the range in which a steady state can be maintained and there was no evidence of any progressive change in the metabolism during the control experiments. The control ranges in Figs. 1 and 2 have been arbitrarily limited by the averages of the highest and the lowest oxygen consumptions and carbon dioxide outputs obtained in each of the four control experiments. In Fig. 1 the range for the oxygen consumption is 11 p.c. of the average oxygen consumption at this level of exercise and the carbon dioxide range is 12 p.c. of the average carbon dioxide production. For Fig. 2 the corresponding values are 12 and 13 p.c. In thirty estimations of basal metabolism on the same subject the corresponding ranges were 12 and 16 p.c. It appears therefore that the variability of the oxygen consumption and of the carbon dioxide output is, if anything, slightly less during a steady state of mild exercise than it is in the basal state.

Summary

The duration of the recovery period following standing running at top speed for 30 sec. has been determined using a steady state of mild exercise as a base line instead of the basal state of complete rest. Recovery is complete in from 20 to 45 min. after exercise of this type. Recovery to the basal state, after similar exercise, takes at least 90 min.

It is a pleasure to acknowledge our indebtedness to Prof. A. V. Hill for suggesting this problem. Mr T. Brodie served faithfully as subject for all the experiments. Mr J. G. Truax rendered valuable assistance in the performance of many of the gas analyses.

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Since this paper was written a report has appeared (Newman, E. V., Dill, D. B., Edwards, H. T. and Webster, F. A. [1937], *Amer. J. Physiol.* **118**, 457) of the results of somewhat similar experiments in which the rate of removal of lactic acid from the blood following violent exercise of short duration was followed during continued mild exercise. It was found that the rate of removal of lactic acid from the blood was considerably greater during a steady state of mild exercise than at rest. This observation probably explains the more rapid recovery noted in our experiments in which an exercise base line was used.

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Cortical facilitation with electric stimuli.

By E. D. Adrian

In a recent paper on the electrical excitation of the cerebral cortex [Adrian, 1936*b*] it was stated that the activity of superficial elements may be aroused without producing after-effects or spread to neighbouring regions. It has now been found that there may be a distinct facilitating effect. This can be shown with single stimuli or a very short series, but not with the repeated stimulation used previously. It is best seen in animals anæsthetized with dial, where the cortex tends to give regular potential waves at 10 a sec. resembling the Berger waves in man [Bremer, 1935]. In the cat these waves occur spontaneously in groups at intervals of a few seconds, but groups can be started at shorter intervals by single stimuli to the surface of the cortex. The first waves come from the neurones directly excited by the stimulus, but in the course of the discharge fresh neurones become active, so that ultimately the stimulus may determine a period of activity over a wide area. In the rabbit the waves are absent or infrequent when the animal is undisturbed, but a long series can be started either by sensory stimulation (pinching a foot) or by an electrical stimulus to the cortex. Here, too, the stimulus has a widespread effect, and the greatest activity is often 5 mm. or more from the stimulated point. In the monkey under dial the striate area gives waves at 10 a sec. after its activity has been aroused by illuminating the eye [Adrian, 1936*a*] and waves of the same type follow an electric stimulus to the surface of the area.

An analysis of the potential changes shows that they are mainly due to elements in the outer layers of the cortex. These tend to discharge synchronously owing to direct interaction and not because the rhythm is determined by subcortical centres. The waves appear when the level of excitation has risen spontaneously to the point at which discharge must take place, or when it has been raised to that level by afferent impulses or on electrical stimulus, but in the sleep induced by dial the discharge level cannot be maintained for long, even with repeated facilitation.

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The early lesions of vitamin A deficiency.

By J. T. Irving and M. B. Richards

Various workers have observed inco-ordination and paralysis in animals suffering from avitaminosis A, and have found in such cases degeneration in the central and peripheral nervous systems. Many rats on a vitamin A-free diet die, however, without having shown any marked clinical signs of nerve involvement. Richards & Simpson [1934] showed that the weight curve is no reliable criterion of the degree of avitaminosis A in rats, and their work indicated that, in many cases, degenerative changes of the gastro-intestinal tract must have been present before the weight curve began to fall. The present investigation was therefore carried out to determine whether nerve lesions can be detected histologically in the early stages of vitamin A deficiency.

Albino and hooded Lister rats were fed on a vitamin A-free diet from weaning (23 days). In order to ensure that they had no large reserve stores of vitamin A, the mothers were fed on a diet low in vitamin A from the sixteenth day after parturition (about the time young rats start to eat their mothers' food). Positive controls were fed on the same vitamin A-free diet, using the same technique, and were given about 20γ carotene in cottonseed oil daily after weaning.

Animals were killed at intervals from 7 to 42 days after weaning. The C.N.S. was removed and definite levels stained by Marchi's method; our results in this communication deal only with the lower medulla and upper cervical cord.

With one exception none of the animals showed definite signs of loss of weight or of very notable slackening in the growth rate till at least 28 days on the diet. Nevertheless, from 21 days onwards, the animals on the vitamin A-free diet showed a progressive degeneration of certain tracts in the medulla. These tracts lie in the area which would be occupied by the fillet in higher sections; below the pyramidal decussation they become the antero-lateral columns, the pyramidal tracts decussating completely in the rat.

At 21 days, when the animals were still growing, the degeneration was marked only at the motor decussation level and extended but a

short distance into the cervical cord. At 28–31 days, when the growth rate was slackening, the degeneration began slightly higher in the "fillet area", was more intense at the decussation level, and extended farther into the cord. From 35 days onwards the degeneration was still more intense and spread farther up and down the medulla and cord, the posterior columns being involved as well. As these are the only levels we have yet examined, we do not know the further extent of these lesions. The tracts involved are probably the spino-thalamic and spino-tectal.

Animals on the vitamin A-free diet for 7–14 days showed a slight peppering of the same tracts with degenerated fibres. In the positive controls also a few scattered degenerated fibres were frequently observed. This may possibly be due to the fact that, like the minus A rats, the controls got little vitamin A (apart from that in milk) prior to weaning. This slight degeneration was not progressive. Controls killed after 35 days on the diet showed fewer degenerated fibres than those killed at earlier dates.

It is generally held that 10 days have to elapse before degenerated fibres will stain by Marchi's method. If this is also true of this degenerative process, then it would appear that, even in cases of bad loss of weight, the lesions in these rats must have begun while growth was still in progress.

The uniformity with which the condition described occurs in every one of the rats examined is in striking contrast with the variability in pathological condition, and in the occurrence of epithelial metaplasia found in animals in advanced avitaminosis A. The fact that this degeneration in the medulla occurs with such uniformity before the weight curve is affected to any extent, suggests that it is one of the fundamental lesions of vitamin A deficiency.

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Vaso-motor carotid sinus reflexes in experimental hypertension produced by renal ischemia. By J. J. Bouckaert, L. Elaut and C. Heymans

Goldblatt *et al.* [1934] showed that the application of special adjustable clamps to both renal arteries caused a reduction in the blood flow to the kidneys accompanied by persistent elevation of the systolic and diastolic blood pressure. These observations have been confirmed by one of us [Elaut, 1936] and by other investigators.

It is known that under normal conditions the general arterial blood pressure is automatically regulated and buffered by the carotid sinus and aortic arch reflexes [C. Heymans *et al.* 1933]. The question for investigation was whether or not these regulating and buffering reflexes are active in animals with chronic hypertension induced by constriction of the renal arteries.

Three dogs, prepared following Goldblatt's technique, developed permanent hypertension with blood pressure ranging from 180 to 240 mm. Hg. The blood pressure was registered regularly by direct femoral arterial puncture, before and during the period of renal ischemia. Several months after the onset of the hypertension, the vaso-motor reflexes of carotid sinus origin were tested with the dogs under chloralosane anæsthesia. The experiments showed that the usual reflex responses of vaso-constriction and elevation of blood pressure, concurrent with the lowering of endo-vascular pressure in the carotid sinuses, were much more marked in the dogs with hypertension. In these dogs, as a consequence of the reflex vaso-constriction and cardiac acceleration induced by clamping both common carotid arteries, the general arterial blood pressure reached a level of 320 mm. Hg.

These experiments show that this type of chronic arterial hypertension is associated with increased reflex excitability of the vaso-constrictor mechanisms. This hyperexcitability may be due to either central or peripheral nervous or humoral phenomena.

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Some observations on the sympathectomized and vagotomized dog. By J. J. Bouckaert and C. Heymans

In 1934 it was shown by Z. M. Bacq *et al.* that dogs, from which the sympathetic ganglionic chains have been completely removed, jump, run, fight and are resistant to cold and heat, behaving in these respects exactly like normal dogs. Samaan [1935*a*] showed that the sympathectomized dog suffers no reduction in his capacity for muscular exercise. These observations on the sympathectomized dog have been confirmed and broadened recently by Brouha *et al.* [1936].

Jourdan & Nowak [1934, 1936] demonstrated cardio-accelerator fibres in the cervical vagus nerves of dogs. These cardio-accelerator fibres could be stimulated only by rather drastic excitations.

Dogs were prepared according to the following technique: all the fibres of the right vagus nerve going into the thorax, except the recurrent nerve, were cut at the level of the subclavian artery, and both sympathetic ganglionic chains were totally removed in four aseptic operative stages.

Subsequently, the heart rate of the dogs, at rest, was 70–120 beats per min., which was the same rate Samaan [1935, *a*, *b*] observed in sympathectomized dogs with both vagus nerves intact. In our dogs, as in the totally sympathectomized dogs with both vagus nerves intact, emotional stimuli never produced an acceleration of the heart above the automatic rate of 120–140 beats per min. Also intravenous injections of atropine sulphate into the resting unanæsthetized, sympathectomized dogs with one vago-sympathetic nerve cut, produced an acceleration of the heart rate only to 120–140 beats per min. Thus after atropine paralysis of the cardio-inhibitory fibres of the remaining vago-sympathetic nerve, its cardio-accelerator fibres did not act on the heart rate; indeed, the heart frequency after atropine remained within the limits of the automatic rate. In this respect also our dogs behaved like totally sympathectomized dogs with both cervical vagus nerves intact [Samaan, 1935*b*]. Emotional stimuli after atropine also caused no acceleration of the heart in our sympathectomized dogs with the right vagus cut.

After these observations in chronic experiments on the totally sympathectomized and unilaterally vagotomized dogs, the remaining (left) vago-sympathetic nerve was cut, aseptically, in the neck, thus depriving them of both vago-sympathetic cervical nerves, except the right recurrent nerve [Samaan, 1934].

In these totally sympathectomized and vagotomized dogs, at rest or during emotional excitement or after atropine, the heart rate was 120–140 beats per min., which is the same automatic rate seen after atropine in the totally sympathectomized dog with one or both cervical vago-sympathetic nerves intact.

These observations demonstrate: (*a*) that the general behaviour of a dog from which the paravertebral sympathetic chains have been completely removed, with one vagus cut, remains normal; (*b*) that emotional stimulation or injection of atropine will not cause cardiac acceleration above the automatic rate in dogs completely deprived of their sympathetic ganglionic chains and with both cervical vago-sympathetic nerves intact, or with one or both cervical vago-sympathetic

nerves cut; (c) that, at rest and during excitement, the cardio-accelerator fibres included in the cervical vago-sympathetic nerves of the dog have no stimulating influence on the heart rate of the dog deprived completely of its sympathetic ganglionic chains and with both cervical vago-sympathetic nerves intact, or with the right cervical vago-sympathetic nerve cut.

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Adrenaline oxidase. By H. Blaschko, D. Richter and H. Schlossmann. (*From the Physiological Laboratory, Cambridge*)

The oxidative inactivation of adrenaline in extracts of rats' liver has recently been described [Blaschko & Schlossmann, 1936*a*, *b*]. It was found that cyanide is a suitable means of excluding other side reactions such as autoxidation. In 10^{-3} *M* HCN the total oxygen uptake due to adrenaline was one atom of oxygen per molecule of adrenaline. The inactivation was proportional to the amount of oxygen taken up, and was complete when the increased oxygen uptake of the extracts had come to an end. These observations suggest that, in this reaction, adrenaline reacts with one atom of oxygen to give a biologically inactive reaction product. The system responsible for this cyanide-insensitive oxidation had the properties of an enzyme: it was non-dialysable, it was thermolabile, and it showed a marked stereo-specificity. The naturally occurring lævo-rotatory adrenaline was oxidized twice as fast as the dextro-rotatory stereo-isomeride.

The reaction was inhibited by octyl alcohol and urethane; it was not inhibited by cyanide, carbon monoxide or reduced glutathione. These observations show that the enzyme is not identical with catechol oxidase, an enzyme which is also known to oxidize adrenaline. Catechol oxidase is insensitive to narcotics, but is inhibited by cyanide, carbon monoxide [Keilin, 1928] and reduced glutathione [Toscano Rico & Baptista, 1935]. Moreover, the product of the reaction catalyzed by liver extracts still gave the green coloration with ferric chloride characteristic of a catechol derivative.

That in 10^{-3} *M* HCN only one atom of oxygen per molecule of adrenaline is taken up gives a convenient means of identifying the system in tissue extracts. The system was found to be present in liver and kidney of rabbits, rats and guinea-pigs, and in guinea-pigs' intestine. The activity of liver was higher than that of kidney; rats' organs were more active than rabbits' and less active than guinea-pigs' organs. The activity of the extracts was considerable in view of the small amounts of adrenaline usually present in the tissues; extracts from 1 g. of rats' liver inactivated 1 mg. of adrenaline in 15 min., and extracts from 1 g. of guinea-pigs' liver inactivated 1 mg. adrenaline in 8 min.

The system was not found to be present in significant amounts in skeletal muscle (rat), heart (guinea-pig), or spleen (dog, rat). It appeared to be present in small amounts in brain (guinea-pig) and lung (guinea-pig).

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The quantitative effects of antagonistic drugs.

By J. H. Gaddum

Quantitative studies of the antagonism between adrenaline and ergotamine [Gaddum, 1926; Mendez, 1928; Nanda, 1930] and between acetylcholine and atropine [Clark, 1926, 1927] have shown that the effects of drugs of this kind are governed by laws which can be expressed mathematically. If, for example, the effect of adrenaline on rabbit's uterus is plotted against the logarithm of the dose, a symmetrical S-shaped curve is obtained. This may be interpreted on the theory that the muscle contains a number of receptors with different thresholds, and that the logarithms of the thresholds of these receptors are normally distributed [Gaddum, 1926]. This theory would fit the facts whatever the slope of the curve.

It was pointed out by Clark [1926] that if it is assumed that the pharmacological effect is proportional to the amount of drug combining with receptors in the muscle, and that the combination is reversible (or that the combined drug is fairly rapidly destroyed), the shape of the curves may be explained in terms of the laws of mass action. This theory, in its simplest form, dictates the slope of the S-shaped curve, and receives

strong support from the fact that, in the cases which have been studied, acetylcholine and adrenaline have always produced curves of the correct slope.

If similar observations are made in the presence of different concentrations of an antagonistic drug (ergotamine or atropine), and if similar curves, connecting the effect with the logarithm of the dose, are constructed, their slope remains the same. In other words, the dose of the active drug (adrenaline or acetylcholine) must be increased in a constant ratio in order to produce any given effect. In the simplest case this ratio is linearly related to the concentration of the antagonistic drug [Gaddum, 1926]. Clark [1933] has stated that such results can be expressed by the equation $C_1 - C_0 = KC_2^n$, where C_1 and C_2 are the concentrations of the active and antagonistic drugs respectively, and C_0 is the concentration of the active drug which produces the same effect in the absence of the antagonistic drug. In the simplest case $n=1$. This equation is incompatible with the observed fact that when C_2 is constant the above-mentioned ratio C_1/C_0 is constant for effects of different sizes.

A better equation can be derived from an extension of Clark's theory by assuming that the antagonistic drugs act by competing with the active drugs for the receptors and inertly blocking them up. Let y and z be the percentages of the receptors occupied by the active and antagonistic drugs respectively. The percentage of receptors which are free will be $(100-y-z)$. For equilibrium conditions the rate of combination of either drug with the free receptors (which will be proportional to the concentration of the drug and the percentage of the free receptors) will be equal to the rate at which the drug is removed from combination with the receptors (which will be proportional to the percentage of the receptors occupied by the drug). Therefore $K_1C_1\ (100-y-z)=y$ and $K_2C_2\ (100-y-z)=z$, where K_1 and K_2 are constants. Elimination of z gives the equation $K_1C_1=(1+K_2C_2)\frac{y}{100-y}$. In accordance with Clark's theory $y=$ the effect of the drug. This equation agrees with the observed relationship between C_1, C_2 and y in the simplest case.

The more general equation $K_1C_1=(1+K_2C_2^n)\frac{y}{100-y}$ fits the facts in all cases that have been studied. In the case of the action of atropine on the frog's rectus abdominus, $n=1{\cdot}5$ [Clark, 1926]. This might perhaps be interpreted on the theory that on the average 1·5 molecules of atropine must be simultaneously present to block each receptor, but in the case of the action of ergotamine on a guinea-pig's vas deferens, where $n=0{\cdot}5$ [Mendez, 1928], there is no obvious physical interpretation.

The theory under discussion provides an equation which expresses the observed results mathematically, but it involves difficulties. If, for

example, a sufficient concentration of adrenaline is applied to a muscle which has been immersed in ergotamine, a maximal contraction occurs in less than 1 min. On the theory under discussion the adrenaline must have taken the place of molecules of ergotamine which have become dissociated from the receptors during this short time. The combination of ergotamine with and its dissociation from the receptors must be fairly rapid processes. On the other hand, a muscle must be immersed in a solution of ergotamine for at least 1 hour before the action of the drug is complete, and washing out is also slow. The slowness of these processes cannot be due to a slow combination with the receptors, and must be due to some other factor such as slow diffusion through the tissues to the receptors. This view is supported by the fact that the rate of action of the antagonistic drugs is unaffected by the presence of the active drugs in the bath.

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The release of pharmacologically active substances by nerve trunks during electrical stimulation. By J. H. GADDUM, M. A. KHAYYAL and H. RYDIN

It has been found that stimulation of the isolated trunk of a nerve may lead to the liberation of potassium [Cowan, 1934], substances having certain effects similar to those of acetylcholine and adrenaline [Calabro, 1933; Bergami, 1936], and a substance which increases the effect of acetylcholine on eserinized leech muscle [Binet & Minz, 1936]. The release of these substances has been thought to be a consequence of the passage of nervous impulses.

If the cervical vagus of a cat is isolated, immersed in eserinized Locke's solution, and strongly stimulated, a substance is liberated which acts like acetylcholine on eserinized leech muscle. This substance is unstable in alkali, and has comparatively little action on normal leech muscle. These facts are compatible with the conclusion that the substance is acetylcholine.

The largest effects were obtained in experiments in which the portion of the nerve which had actually been in contact with the electrodes was

subsequently immersed for some minutes in the salt solution. In experiments where this portion of the nerve was kept well away from the salt solution, the effects were comparatively small and inconstant, and may have been due to a small uncontrolled leakage of fluid along the nerve. Positive results were obtained only when the stimulation was so strong that visible desiccation and discoloration occurred between the electrodes, and were probably due to heating of the nerve. A similar effect occurred when the nerve was heated by a constant current or a small cautery.

A brief reference to some of these results [Gaddum, 1936] contains the implication that the release of these active substances is a physiological phenomenon. It now seems probable that the experiments are really comparable with the preparation of an extract of the nerve. The release of the active substance is surprising, but does not reveal a physiological mechanism. The main object of this communication is to draw attention to this local phenomenon at the electrodes. The methods used by the workers mentioned above would not discriminate between effects due to the passage of impulses, and effects due to local changes at the electrodes.

The evidence of Binet & Minz for the theory that stimulation of the vagus leads to the formation of a substance which sensitizes tissues to acetylcholine depends partly on experiments similar to those discussed above. They found that if the cervical vagus of a dog was immersed in physiological salt solution and stimulated, it acquired the property of increasing the sensitivity of eserinized leech muscle to acetylcholine. We have repeated this experiment. In some cases the solutions did seem to cause a slight increase in the sensitivity of the muscle, but more frequently they had no effect at all. The result did not seem to be affected in any way by stimulation, with or without immersion of the stimulated portion of the nerve. We are indebted to Prof. Binet and Dr Minz for instructing us in certain details of their technique which have not been made public. In spite of this assistance we have been unable to obtain convincing results.

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The nervous impulse as an electrical stimulus. By A. L. HODGKIN.
(*From the Physiological Laboratory, Cambridge*)

This communication is concerned with the way in which an active section of a nerve fibre excites an adjacent part. The experimental method depends upon the observation that a blocked impulse can sum with a sub-threshold electric shock. Blair & Erlanger [1936] found this kind of summation in nerves blocked by anodal polarization; in my experiments cold or compression blocks have been used. Summation can be observed in the following way. A frog's sciatic nerve is blocked by local cold or pressure. A maximal shock S_1 is applied to A, and a sub-threshold shock S_2 to B (Fig. 1). Neither shock alone produces a response at C, but if they are combined, so that S_2 follows S_1 by one or two milliseconds, impulses are set up and can be detected at C. Clearly a volley of blocked

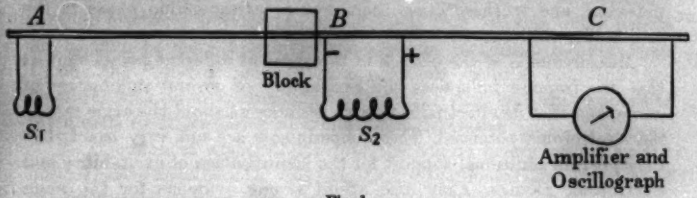

Fig. 1.

impulses increases the excitability at B and enables S_2 to excite. The increase lasts for a few milliseconds, and may involve a decrease of 70 p.c. in the electrical threshold.

The summation may be explained by supposing that the action potential sets up local electric circuits which spread through the blocked region. If this is correct, the change in excitability should be associated with some kind of electrical disturbance. This anticipation has been verified; for the increase in excitability is invariably accompanied by a characteristic potential wave. The experimental observation is that an electrical change can be recorded from the stretch of nerve beyond the block, although no impulses are transmitted through it. This may be distinguished from an ordinary action potential by the fact that it declines exponentially along the nerve. The exponential gradient produced by an impulse was compared directly with the potential gradient in the extrapolar region of a cathode. A potential difference with a wave form similar to the action potential was applied to the blocked section, and electrotonus was recorded from the stretch of nerve distal to the

block. Eleven experiments were performed, and in every case the gradient of electrotonic potential was found to be very similar to the gradient produced by an impulse. Both potentials are abolished by crushing the nerve between block and recording electrodes. The conclusion from these experiments is that the potential set up by a blocked impulse is essentially similar to catelectrotonus.

The constant association of electrical and excitability change suggests that the spread of catelectrotonus is responsible for the increase in excitability. More definite evidence was obtained from an investigation of the time relations of the two processes. It was found that the time course of the increase in excitability was always similar to that of the potential. At a cold block both potential and excitability change lasted from 5 to 10 msec., while at a pressure block they sometimes occupied only 3 msec. The main wave was occasionally followed by a large after-potential, and in these cases there was a corresponding phase in the excitability time curve.

Measurements of the change in threshold at different points showed that the impulse influences the excitability of several millimetres of nerve, and that its effect falls off with distance at about the same rate as the electrotonic potential. These experiments are not very exact, but they provide additional support for the identification of excitability and electrotonic change. They also afford strong evidence for the more general proposition that the increase in excitability depends upon electric currents; for electricity is the only agent which is likely to affect a distance of several millimetres in the time available.

The experiments described here indicate (*a*) that an action potential sets up a transient gradient of catelectrotonus in the inactive parts of a nerve fibre, and (*b*) that this can produce large changes in excitability. The general conclusion is that the spread of catelectrotonus must play an important part in nervous transmission.

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Actions of acetylcholine on denervated mammalian and on frog's muscle. By G. L. Brown. (*From the National Institute for Medical Research, London, N.W.* 3)

1. The response to acetylcholine of the denervated gastrocnemius of the cat has been examined by the method of close arteria injection.

The injection of small doses (0·001–0·1 γ in 0·5 c.c. saline) is followed by a "quick" response, superficially little different from that elicited from normal muscle. With a larger dose (0·5–2·5 γ) the "quick" response is followed by a more slowly developing contracture. When still larger doses are given (2·5–10 γ), the "quick" response is curtailed and passes with little interruption into the contracture, the whole response showing a very nearly smooth curve.

The electrical activity of the muscles has been recorded with concentric needle electrodes, amplifier and oscillograph. Denervated muscle is in a state of constant fibrillary activity, which appears as an irregular series of diphasic potentials. The "quick" response to acetylcholine is accompanied by an outburst of action potentials like that occurring in normal muscle, but persisting very much longer. When a larger dose of acetylcholine is given, the electrical activity accompanying the "quick" portion of the response is abruptly cut off and, during the slow contracture, no electrical activity can be detected. This "silence" may persist for 10 min. or more before the spontaneous fibrillation recommences. During the "silence" acetylcholine injection produces only the contracture, with no accompanying electrical activity, and the response of the muscle to direct electrical excitation is depressed.

2. Injection of acetylcholine into the sciatic artery supplying the normal gastrocnemius of the frog has produced responses similar to those of the denervated mammalian muscle. Injection of 0·1–1 γ (in 0·1 c.c.) causes a contraction associated with a long-lasting outburst of asynchronous action potentials. A larger dose (5–10 γ) causes an initial quick response, associated with electrical changes, followed by a contracture which abruptly terminates the electrical outburst.

Acetylcholine, therefore, can set up propagated contractions in denervated mammalian and in frog's muscle. These contractions are suppressed by the contracture which occurs if sufficiently large amounts are given. The contracture also depresses the response of the muscle to direct electrical excitation.

The inorganic constitution of the anterior retractor of the byssus of *Mytilus edulis*. By INDERJIT SINGH. (*From the Physiological Laboratory, Cambridge*)

The percentage of water in eighteen muscles ranged from 74 to 82. This was estimated by weighing the muscle before and after heating to 110° C. in an air-oven. Subsequently the residue was ashed with nitric-

sulphuric acid mixture and the ash weighed to determine the total inorganic cations as sulphates. In other experiments the wet muscle was ashed directly.

In the muscle ash sodium was estimated by Eisenman's modification [1927] of the titrimetric method of Kramer & Gittleman [1924], and potassium was estimated by Brigg's diazo-cobaltinitrite microcolorimetric method [1923] as described by Peters & van Slyke [1932]. After a few preliminary analyses the methods were tested by determinations of amounts of sodium and potassium (A.R. salts) comparable with those found in single muscles. Usually, 0·1 mg. of potassium could be determined to within about ±1 p.c., but when 0·3 mg. of sodium was determined, the amount found was about 10 p.c. too small, and with smaller amounts the percentage found was still lower. After further tests with larger amounts of sodium, it was found that 0·4–1 mg. could be determined to within about ±2 p.c. For subsequent analyses three or four muscles were ashed together, one-quarter or one-half of the ash taken for the potassium estimation and the remaining three-quarters or one-half for the sodium estimation. In addition to the errors associated with the chemical analyses, there were the errors attendant on the weighing of wet tissue, so that the final accuracy of the sodium and potassium determinations in muscle was about ±5 p.c.

The sodium content of freshly dissected muscles from winter specimens of *Mytilus*, which had just arrived from Plymouth or which had been in the laboratory for about a day, ranged from 3 to 4·4 mg. per g. of wet muscle; the potassium content from 1·3 to 1·6 mg. per g. (six analyses). The keeping of these animals in a refrigerator caused the anterior retractor to gain sodium. In *Mytilus*, stored at 2–5° C. for a fortnight or 3 weeks, values up to 9·5 mg. per g. of wet muscle were found (twenty analyses).

Striated muscle contains much potassium and little sodium [cf. Fenn, 1936], but unstriated muscle has been found to contain relatively more sodium and less potassium [cf. Evans, 1926; Fenn, 1936]. In the unstriated retractor of *Mytilus* the sodium content appears always to exceed the potassium content, both when the muscles are taken from fresh animals, and animals kept in a refrigerator.

If *Mytilus* muscle is isotonic with sea water, and dissolved electrolytes are wholly responsible for the osmotic pressure within the fibres, their electrolyte content should be 0·44 milliequivalent per g. of muscle (allowing for a water content of 78 p.c.); in fact, the content of sodium and potassium together amounts only to 0·18–0·24 milliequivalent per g.

in fresh muscle, though it approaches the expected value more closely in muscles from animals which have been cooled.

To check the above results and to determine whether any other inorganic cation was present in appreciable amounts, the total cations were determined as sulphates on a second series of muscles, both from recently caught and from cooled animals. The weights of the sulphates ranged from 16·1 to 25·9 mg. per g., corresponding to no greater amounts of cations than had been found in the earlier experiments.

In a third series of analyses the total base, inorganic and organic, that could be recovered from single muscles by electro-dialyzing by Adair & Keys's method [1934] was determined titrimetrically. The values ranged from 0·226 to 0·259 milliequivalent per g. The efficiency of the dialysis was tested by solutions containing known amounts of the chlorides of the cations expected. With sodium, potassium, ammonium and lithium, 98–99 p.c. was recovered; with barium, strontium and calcium, 90–96 p.c. was recovered; with magnesium only about 50 p.c. was recovered.

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The distribution of water in the sexual skin of monkeys.

By R. B. Fisher and S. Zuckerman

The water balance in the pig-tailed macaque varies cyclically in accordance with the menstrual cycle and the cycle of sexual-skin swelling [Krohn & Zuckerman, 1936]. The following observations relate to the state of water in the sexual skin at different stages of swelling. By sexual skin is meant both the circumgenital swelling which occurs normally in certain females and the corresponding swelling which can be produced experimentally in some male monkeys. Observations on seven male rhesus macaques in which scrotal swelling was induced by the injection of œstrone are collected in Table I, and observations on the normal sexual skin of three female hamadryas baboons are given in Table II.

TABLE I. Observations on male rhesus monkeys

Specimen	Oestrone (γ per diem × days)	Days after beginning of subsidence of swelling	Days after beginning of swelling	p.c. exudate in fat-free specimen	p.c. water in drained fat-free specimen	Freezing point depression of exudate (Δ_e)	Freezing point depression of serum (Δ_s)	$\Delta_s - \Delta_e$
153	100 × 22	—	12	—	—	0·697	0·616	−0·079
174 S[1]	100 × 21	—	15	77	77	0·825	0·721	+0·097
174 B[2]	100 × 21	—	8	36	82	0·655	0·721	+0·067
175	100 × 14	1	8	66	70	0·618	0·655	+0·037
173	200 × 14	2	7	37	72	0·589	0·654	+0·065
155	100 × 37	8	17	80	46	0·606	0·605	+0·001
151	100 × 37	18	26	—	—	0·596	0·601	+0·005
161	100 × 14	4[3]	5	53	46	0·598	0·604	+0·006

[1] Scrotal swelling.
[2] Swelling of subcutaneous tissues of lower part of back.
[3] Very slight swelling.

TABLE II. Observations on female hamadryas baboons

Specimen	Days after beginning of subsidence of swelling	Days after beginning of swelling	Freezing point depression of serum (Δ_s)	Freezing point depression of exudate (Δ_e)	$\Delta_s - \Delta_e$
EU 9	—	10	0·656	0·968	−0·312
EU 3	1½	49	0·715	0·683	+0·032
EU 7	5	17	0·577	0·590	−0·013

Exudates of the sexual skin were obtained post-mortem by cutting up the swollen tissue sufficiently to allow ready drainage of the exudate, which was filtered through muslin. A sample of the exudate was taken for the determination of its freezing point after 30–40 min. drainage, and drainage was then allowed to continue overnight in cold store. The total exudate and the drained tissue were weighed, and then exhaustively extracted with boiling alcohol. The alcohol extract was taken to dryness, and the residue, which was taken to represent the fat of the original specimen, was weighed. The tissue left after alcohol extraction was also dried and weighed. The difference between the weight of this final tissue residue and the weight of the drained tissue (less the fat) was taken as a measure of the water content of the drained tissue. In this way rough measures of the proportions of intercellular fluid (exudate) to total tissue, and of intracellular fluid (water content of drained tissue) to total cellular tissue were obtained.

Table I shows that in specimens taken within 2 days of the beginning of the subsidence of the swelling, the water held in the drained tissue constitutes 70–80 p.c. of its weight, whereas, in specimens taken later on, the water content appears to be in the region of 50 p.c. This is consistent with the view that the swelling is partly due to an increased imbibition

of water by the tissue. If imbibition is as rapid as is suggested by the normal rate of swelling, it might be expected that the withdrawal of water by the cells from the intercellular fluid will occur more rapidly than it can be made up by diffusion from the plasma, and that, in consequence, the osmotic pressure of the exudate should exceed that of the serum. This condition will probably persist in some degree for some time after syneresis begins, to be followed, when syneresis is taking place rapidly, by a dilution of the tissue fluid by the liberated water. Finally, at some stage during the absorption of the exudate, equilibrium between plasma and tissue fluid should be established, and their osmotic pressures should be identical. In Table I all these three stages are to be seen, and it is of particular interest that correspondence between the osmotic pressures of serum and exudate appears to occur only when the water content of the drained tissue has dropped to a level corresponding to that to be expected in normal subcutaneous tissue.

The specimen EU 9 in Table II is particularly important in view of the very large excess of exudate osmotic pressure over the osmotic pressure of the serum. This observation alone suggests that the sexual-skin swelling is at least partly an imbibition phenomenon.

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Body temperature and oxygen poisoning.

By J. Argyll Campbell

P. Bert [1878] demonstrated that mammals are poisoned by oxygen at pressures above 4 atmospheres. One of the objective symptoms is a marked fall in body temperature, sometimes by as much as 10° C. This fall in temperature has been frequently confirmed and is regarded as being due to the depression of metabolic processes. Recently some evidence has been obtained that this fall of temperature is protective. Thus if the body temperature is prevented from falling greatly by keeping the external temperature between 31–35° C., the poisoning is markedly enhanced. By exposing white rats to 6 atmospheres of oxygen for 30 min. and decompressing them slowly for 20 min.—to prevent bubble formation in the tissues—this influence of temperature is clearly demonstrated. Thus at 31–35° C. there are rarely any survivors (one out of ten), but at 21–27° C. there are rarely any deaths (one out of ten). Observations

of longer duration give similar but less marked differences. The experiments were carried out in the small compression chamber at Messrs Siebe Gorman's premises. Soda-lime was placed in the chamber, and there was also a through ventilation of oxygen to keep the carbon dioxide at a minimum. Analyses showed that this was present in traces only.

Effect of external temperature on oxygen poisoning (white rats)

Expt. no.	Pressure O_2 atm.	Duration of compression; min.	Time for decompression; min.	Number of animals used	Survivors at external temperature	
					33° C.	24° C.
1	5	60	30	5 (S.)	0	5
				5 (N.S.)	0	—
2	6	60	36	12 (S.)	—	4
				12 (N.S.)	—	1
3	6	40	20	3 (S.)	0	3
				6 (N.S.)	0	1
4	6	30	20	5 (S.)	1	4
				5 (N.S.)	0	5
5	6	30	20	5 (S.)	2	4
				5 (N.S.)	0	4
				Survivors *Totals*	3 out of 39	31 out of 58
			Survivors	Starved 60 hr. (S.)	3 out of 18	20 out of 30
				Non-starved (N.S.)	0 out of 21	11 out of 28

Other factors which have been found to influence oxygen poisoning are (*a*) carbon dioxide pressure in the tissues [Gesell, 1923; Campbell, 1929; Hill, 1933; and others]; increase of this pressure, e.g. by breathing 5 p.c. carbon dioxide, increases mortality; (*b*) food; starvation lessens mortality [Almeida, 1934]. Recently we have confirmed Almeida's findings, but starvation does not appear so effective as the temperature factor.

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A collapsible face-tent for oxygen administration.

By J. Argyll Campbell

A box mask for administration of oxygen was demonstrated recently [Campbell, 1936]. The collapsible face-tent is similar in size (15 × 12 × 7 cm.), shape and principle, but whereas the box mask is made

from cardboard or aluminium, the face-tent consists of a wire frame covered with closely woven linen. The aluminium box mask weighs about 100 g. and the face-tent less than 50 g., so that it is of value for frail patients. Also the linen may be moistened so that the evaporation of this moisture will cool the face. Further, the wire frame is collapsible and the apparatus thus occupies less space in transport. With a flow of 6 litres of oxygen per min., the alveolar oxygen pressure may be kept at 50 p.c. of an atmosphere. This is high enough for most cases, e.g. pneumonia. For cases of carbon monoxide poisoning the alveolar oxygen pressure may be raised to 80 p.c. by hanging a curtain of lint 4½ in. long from the bottom of the tent, and using a flow of 8 litres of oxygen per min. As with the box mask, a Bobbin flow meter and standard automatic regulator for the oxygen cylinder are required. Makers: Messrs Siebe Gorman & Co. Ltd., London, S.E. 1.

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The development of the "righting" movements in the fœtal sheep. By J. Barcroft and D. H. Barron

Between the forty-seventh and fiftieth days of intrauterine life (total gestation period of sheep is about 150 days) the spontaneous and induced activity of the fœtus resembles an attempt to right itself and to stand up. We have followed these movements as they are built from the time of the appearance of their first component when the fœtus is about 38 days old as follows:

Fœtus, age 38 *days.* The head is rotated when the snout is stimulated. The limbs move only passively.

Fœtus, age 40 *days.* This fœtus exhibited no spontaneous activity; when stimulated on the snout with a probe the head is moved slightly backwards and away from the stimulus. In addition, the dorsal trunk muscles contract at the same time. These contractions are not sustained. The limbs do not yet appear in these movements.

Fœtus, age 41 *days.* The movements have now spread to a sustained lateral flexion of the neck, with a very slight head rotation, and the forelegs are extended slightly and together. The head is raised away from

the solid surface on which the fœtus lies. Sustained movements appear for the first time. The hindlegs are not yet involved.

Fœtus, age 42 *days.* This fœtus is introduced to show that at this age the foreleg movement is associated with the lateral flexion of the neck when the area of the Vth nerve is stimulated electrically. This movement, expanded and strengthened, is the basic part of the "righting" complex.

Fœtus, age 45 *days.* The forelimbs extend slightly and the hindlegs flex stiffly at the hips: these movements are not co-ordinated with head rotation, which is now quite limited.

Fœtus, age 47 *days.* The head and limb movements are of greater amplitude than at 45 days.

Fœtus, age 48 *days.* This fœtus is outside the amniotic; the movements are still more extensive. The neck is flexed more markedly laterally, and hindlimbs are flexed more strongly on the belly.

Fœtus, age 49 *days.* The "righting" movements appear best developed at this age and have not yet been suppressed along with general activity. These movements, however, do not appear *in utero*, but only when the fœtus is lying on a solid surface.

Fœtus, age 52 *days.* At this age the activity becomes suppressed somewhat: the fœtus does not attempt to "right" itself though rotation of the head is still resisted.

Fœtus, age 64 *days.* Though younger fœtuses actively attempt to stand, at this age activity appears to be suppressed and the fœtus resists attempts to arouse it, except when the umbilical cord is compressed. Even then it does not attempt "righting" or "standing". The uterus has been transilluminated but the fœtus did not appear to be oriented in relation to gravity.

These points are illustrated by a film.

An installation for producing a dry atmosphere.

By E. P. Poulton

The drying apparatus consists of a motor fan that drives about 15 cu. ft. per min. of air from the room, at first through a filter to remove dust and then through a receptacle containing 40 lb. of silica gel which absorbs moisture, through a second filter to keep back particles of silica and finally through a sleeve into a bed tent, out of which it escapes

through a second opening. After 12 hours or more when the silica gel is exhausted, it is reactivated by turning on the fan while the silica is heated electrically to 220° F., a process that takes about 6 hours. I am indebted to Silica Gel Ltd. for providing the apparatus. The humidity has been measured with a hair hygometer and also with a sling psychrometer containing dry- and wet-bulb thermometers, to control the former. With room temperature 68° F. at 9.30 a.m. and humidity 51 p.c., the air in the tent had a temperature of 78–80° F., and a humidity of under 16 p.c. for 8 hours.

Action of prostigmine on spinal cord in man. By M. KREMER, H. E. S. PEARSON and SAMSON WRIGHT. (*From the Department of Physiology, Middlesex Hospital Medical School*)

The action of prostigmine on the human spinal cord when injected intrathecally in doses of 1–1·5 mg. has been studied in eight patients (one cerebral diplegia, one spastic paraplegia due to spinal tumour, and six hemiplegia). The film illustrates some of the effects typically produced in a case of hemiplegia. The clinical details are as follows:

F.K. Male, age 65. Right-sided hemiplegia (due to cerebral thrombosis), 17 months

Clinical state.

	Arms			Head
Side	Power	Tone	Reflexes	
Right	Weak	++	++	Slight weakness lower half of face. Speech slightly affected
Left	Normal	Normal	Brisk	Normal

	Legs					
Side	Power	Tone	A.C.	A.J.	K.J.	Plantar
Right	Weak	++	++ Sustained	+	++	Extensor
Left	Normal	Normal	—	Normal	Brisk	Flexor

Abdominal reflexes present in all four quadrants.

[A.C. = ankle clonus; A.J., K.J. = ankle jerk, knee jerk; Plantar = plantar reflex.]

At 11.45 a.m. inject 1/50 gr. atropine subcutaneously and 1 mg. prostigmine intrathecally (by lumbar puncture).

12 noon (15 min.). No change.

12.15 p.m. (30 min.). Ankle jerk and knee jerk diminished on left side. Other signs unchanged.

12.40 p.m. (55 min.). Patient drowsy and quiet. Sensation unimpaired.

	Arms			
Side	Power	Tone	Reflexes	Head
Right	Unchanged	Slightly diminished	Unchanged	Speech more indistinct
Left	Normal	Normal	Brisk	Normal

	Legs					
Side	Power	Tone	A.C.	A.J.	K.J.	Plantar
Right	Diminished	Absent	Absent	Weak	Weak	Absent
Left	Normal	Absent	—	Absent	Absent	Flexor

Abdominal reflexes: lost in both lower quadrants and diminished in both upper quadrants.

1.45 p.m. (2 hours). Intense pallor face and neck. Vomited. Very drowsy; looked collapsed. Pulse 60. Right side of face puffs out with respiration.

Inject 1/100 gr. atropine and 4 c.c. coramine.

3.45 p.m. (4 hours).

	Arms			
Side	Power	Tone	Reflexes	Head
Right	Nil	Nil	Nil	Face very weak. Not puffed out with respiration. Tongue deviates to right. Speech very indistinct
Left	Normal	Nil	Nil	Normal

	Legs		
Side	Power	Tone	Reflexes
Right	Weak	Nil	Nil
Left	Normal	Nil	Nil

Colour better. Face warmer. Still drowsy. Still feels sick.

Bladder distended but unable to empty it.

4.20 p.m. (4 hr. 35 min.). More awake. Recovery beginning in right leg: return of some power and unsustained ankle clonus. Still cannot pass urine.

5.15 p.m. (5½ hours).

	Arms			
Side	Power	Tone	Reflexes	Head
Right	Wrist movements present	Triceps +	Triceps J. +	As at 3.45 p.m.
Left	Normal	Normal	Triceps J. +	Normal

	Legs		
Side	Power	Tone	Reflexes
Right	Improving	Slight	Ankle clonus sustained Plantar reflex feeble flexor
Left	Normal	Normal	Plantar reflex normal

5.45 p.m. (6 hours).

	Arms			
Side	Power	Tone	Reflexes	Head
Right	Elbow flexion present	Triceps +	Triceps J. + Supinator J. +	Speech better. Otherwise as before
Left	Normal	Normal	Triceps J. + Biceps J. ±	Normal

	Legs					
Side	Power	Tone	A.C.	A.J.	K.J.	Plantar
Right	Improving	Quadriceps increased	Sustained	Weak	Weak	Feeble flexor
Left	Normal	Normal	—	None	None	Normal

Much more awake. General condition better. Catheterized (20 oz. urine).

7.30 p.m. (7¾ hours). General condition good. Right leg: back to pre-injection state. Right arm: still partially relaxed but all tendon jerks brisk. Speech less indistinct. Left side: power good but all tendon jerks feeble.

Next morning (21 hours). Complete return to pre-injection state, but had freer voluntary movement in right arm.

Sensation was unimpaired throughout the experimental period.

PROCEEDINGS

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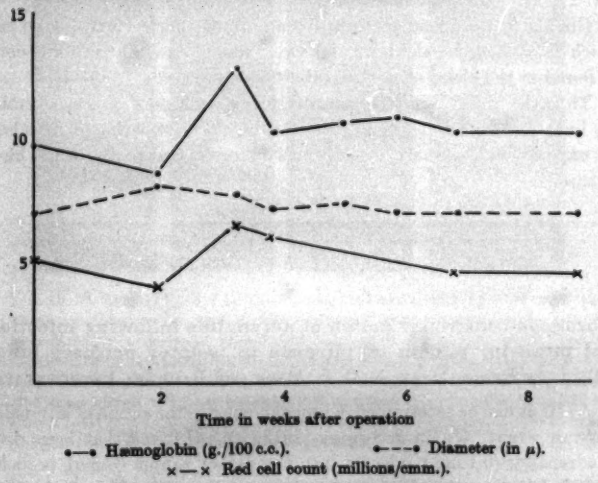
16 *January* 1937

The carotid sinus and blood regeneration.

By A. L. Latner

It is generally suggested that the increased hæmoglobin content and raised red cell count of anoxia are due to a direct action on the bone marrow. These experiments seem to demonstrate that the bone marrow is influenced through the medium of the carotid sinus.



The carotid sinuses were removed and the aortic arch denervated in each of six rabbits by the two-stage operation of Kremer *et al.* [1933]. The hæmoglobin content and the red cell count were determined just

before the second operation. Readings were subsequently renewed 2 weeks later, and then taken at more or less regular intervals.

In practically all cases it was found that the first reading after the operation showed that both the hæmoglobin and red cell count had fallen to a lower level than initially, the colour index usually being above one. Both readings now rose and eventually reached a peak markedly above the initial levels. Eventually they fell to a constant level, which in no case was the same as the original. Readings of the cell diameters also showed variations. The above graph illustrates the readings of one particular case.

The initial variations seem to show that the carotid sinus plays some part in the control of the normal blood picture. The final constancy of the readings seems to indicate that some other controlling mechanism has come into play. This was supported by the blood picture, taken in four of the animals. These all showed the presence of megaloblasts, marked polychromasia and apparently an altered white cell picture. The reticulocyte count in each case was in the neighbourhood of 7 p.c. (normal value is 1–2 p.c.).

Obviously the blood picture was abnormal, particularly as no cells which resembled megaloblasts and which might lead to confusion, could be found in the blood of normal rabbits.

Thus the carotid sinus apparently plays some part in the control of the bone marrow. It is intended in a subsequent experiment to submit the experimental animals to conditions of anoxia and to follow the blood picture.

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A broncho-constrictor action of adrenaline following injections of pituitrin, pitocin or pitressin in isolated perfused lungs.

By I. de Burgh Daly, E. G. L. Mark and Barbara Petrovskaia

As far as can be ascertained, a broncho-constrictor action of adrenaline is a rare event. Golla & Symes [1913, 1913*a*] found that large doses of adrenaline (0·1 mg.) injected into the cat or rabbit caused broncho-constriction, broncho-dilatation only occurring if the bronchi were previously constricted. Our experience in over 100 experiments on the dog is that adrenaline (2γ upwards) almost invariably produces broncho-dilatation, especially when the bronchi are constricted by pilocarpine,

acetylcholine, histamine or ergotoxine. In not more than three experiments has a broncho-constrictor effect been noted, and then only of sufficient degree to reduce the tidal air by 2 p.c. or less: in these cases the constrictor effect occurred early on in the experiment.

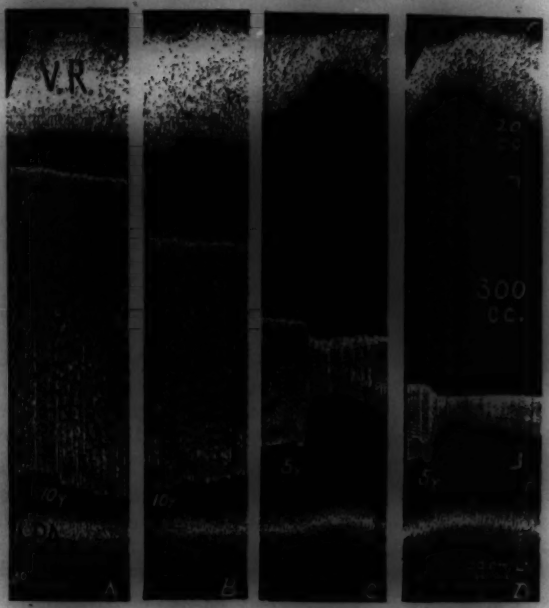

Fig. 1. 4. xii. 36. Dog, 17 kg. I.P.L. Total amount of blood circulating = 500 c.c. Negative pressure ventilation. Top tracing = blood volume of lungs; an upward movement of the lever indicates a decrease: second = tidal air: third = pulmonary arterial pressure: fourth = 30 sec.. *A* = adrenaline 10γ at 1.55 p.m. At 2.15 p.m. the steady infusion into the circulation of 0·03 I.U. pituitrin per min. was started. *B* = adrenaline 10γ at 2.55 p.m. after the addition of 1·2 I.U. pituitrin (P.D. and Co.). *C* = adrenaline 5γ at 3.25 p.m. after a total of 2·1 I.U. pituitrin has been added to the circulation. *D* = adrenaline 5γ at 3.37 p.m. after a total of 2·46 I.U. pituitrin.

In a series of experiments on isolated perfused lungs in which pituitrin, pitressin or pitocin had been injected in order to determine their action on the vascular tone, it was found that these extracts reversed the usual broncho-dilator action of adrenaline to broncho-constrictor. So far, the

smallest doses of pituitrin, pitressin and pitocin found to cause this reversal of adrenaline action have been 1·2, 2·5 and 5 I.U. respectively, but the amount of the extracts necessary varied from one lung preparation to another, and we have yet to discover the most favourable conditions for the effect to take place. The amount of blood circulating in the perfusion system in our experiments is approximately 500 c.c., and therefore the minimal effective concentration of pituitrin, pitressin and pitocin is 2·4, 5 and 10 milliunits per c.c. blood respectively.

The broncho-constrictor action of adrenaline following the administration of extracts of the posterior lobe is reversed by ergotoxine so that adrenaline produces its normal broncho-dilator action in ergotoxinized and pituitrinized preparations.

All three extracts potentiate the pressor action of adrenaline on the pulmonary vascular bed.

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The ultra-high frequency conductivity of tissues.

By J. B. Bateman, D. T. Harris, E. May and H. Rosenberg

It can easily be shown theoretically, and it has been established experimentally, that the heating of homogeneous substances of equal dielectric constant in the condenser field at a given frequency has a maximum value for a certain conductivity. The conductivity for which this maximum heating occurs is a simple function of frequency and dielectric constant, being defined, in the simplest possible case, by the equation $\kappa = 8{\cdot}85.10^{-14}.\omega\epsilon$ (κ = specific conductivity in ohm^{-1} cm.$^{-1}$; ω = circular frequency; ϵ = dielectric constant). This equation has often been used in calculations supporting the contention that, by suitable choice of frequency, the heating of a given element in a heterogeneous conducting body placed in the condenser field could be favoured, and in principle the possibility of such selective heating must be correct. When applied to aggregates of tissues, however, these calculations are largely vitiated by the fact that the values of κ for the various tissues have been commonly assumed to be those measured at low frequencies, although it is sufficiently well known that, at higher frequencies, capacitative barriers between various parts of the tissues are bridged, with a resulting increase in effective conductivity. The simple equation assumes, moreover, that κ is

independent of frequency; although it is quite conceivable that, even when all membrane barriers have been bridged, dipole absorption may render the assumption incorrect (e.g. in the case of nervous tissue, consisting largely of amphoteric substances).

The present measurements are intended to supply values of κ for tissues at wave-lengths between 4·7 and 0·6 m. The tissues, in some cases minced, were placed in Pyrex tubes in the terminating loop (voltage node) of a Lecher system in which standing waves were set up by inductive coupling with the transmitter ($\lambda > 2$ m., two ESW 501 valves in push-pull; $\lambda = 2$–0·6 m., Marconi 4-split magnetron, E 639, both battery-fed). The length of the system was adjusted to resonance by a distant screw with a vernier reading to 0·05 mm., and the resonance amplitude recorded by means of a Cambridge vacuo-junction, placed at the position of maximum current, and a Downing moving-coil galvanometer. This measurement alternated with measurements on a standard salt solution which served as a check on the output of the transmitter. Other determinations were made subsequently with a series of NaCl solutions; κ for these solutions was found in an ordinary Kohlrausch vessel at 1000 hz., and a calibration curve constructed by plotting resonance amplitude at wave-length λ against κ at 1000 hz. κ for the tissue, at wave-length λ, could then be obtained by interpolation. All measurements were made at room temperature. The tissue conductivity at 1000 hz. was also measured by packing into an ebonite trough with platinized Ag electrodes.

The results of our measurements at a single wave-length (2·3 m.) are given in Table I, together with the values of κ at 1000 hz. for the same specimen.

TABLE I

Date	Tissue	$10^3\,\kappa_{2\cdot3\,\mathrm{m.}}$	$10^3\,\kappa_{1000\,\mathrm{hz.}}$
16. ix. 36	Ox hemispheres	3·86	1·26
30. ix. 36	Ox liver	5·35	0·99
4. ix. 36	Dog liver	5·15	1·04
4. ix. 36	Dog liver, minced	5·50	2·14
2. ix. 36	Ox kidney	8·00	3·00
9. ix. 36	Muscle (rump steak)	6·40	6·23
9. ix. 36	Muscle, minced	6·36	6·47
2. ix. 36	Ox fat (kidney)	1·72	~0·06
9. ix. 36	Ox blood (defibrinated)	8·25	5·07

To these data the following additions and comments may be made: (i) In the tissues studied κ was practically independent of frequency over the range $\lambda = 4{\cdot}7 - 2{\cdot}3$ m. Thus our measurements do not permit any distinction to be made between electrolytic conductivity and that due to polar substances. (ii) Between 0·6 and 1·4 m. anomalies occur, which require further elucidation. (iii) The fat extracted from adipose tissue by heating to 100° C. had a conductivity at 2·3 m. of only $0{\cdot}25.10^{-3}$. It

must be inferred that the high value obtained for the intact tissue is due mainly to blood and structural elements. (iv) The inferences to be drawn from these revised values of κ have been illustrated by calculating the heat produced in each of the tissues when placed singly, in the form of a sheet 1 cm. thick, between the plates of a condenser 2 cm. apart. The calculation makes it clear that, although an "optimal" wave-length must exist, at which each tissue is heated selectively, only a rather slight degree of selectivity is to be anticipated. In the much more complex practical case of a living body placed in the condenser field, the effects of this selectivity are likely to be masked by other factors. Fat, with its low dielectric constant and low specific heat, is an exception to this general statement; at all wave-lengths considered it should be heated several times more rapidly than other tissues. Even in this case, however, other factors are of such importance that, according to Coulter & Carter [1936], the subcutaneous tissue of the living human arm is heated somewhat less, between 6 and 24 m., than is the quadriceps extensor muscle.

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The effect of phloridzine on the elimination of dyestuffs in the kidney. By P. Ellinger and A. Lambrechts

In order to throw new light on the mechanism of the glycosuric action of phloridzine, we examined with the intravital microscope the behaviour of fluorescine, trypaflavine and rhodamine B.S. on the kidneys of winter frogs poisoned with phloridzine. We compared this with their behaviour on kidneys of normal winter frogs which was either known from earlier experiments of Ellinger & Hirt or had been tested previously. Winter frogs narcotized with urethane were poisoned with 5 mg. of phloridzine. 15–30 min. later the kidneys were exposed and observed with the intravital microscope. Then 1–2 mg. of one of the dyes dissolved in Ringer's solution were injected into a leg lymph sac.

The kidney of the normal winter frog eliminates plenty of fluorescine into the glomerulus. In the proximal tubules some of the dye is reabsorbed and the epithelial cells become stained. In the kidney of the winter frog poisoned by phloridzine, however, the fluorescine eliminated into the glomerulus is not reabsorbed in the proximal tubules and their epithelium does not become stained. The behaviour of fluorescine towards the other parts of the urinary tract is not affected by phloridzine poisoning.

The behaviour of trypaflavine on the kidney of the winter frog is not affected by phloridzine. But there is no reabsorption of trypaflavine in the proximal tubules of the normal frog, and the diffuse staining of their epithelial cells takes place not from the lumen but from the lymph spaces which remain dark.

Rhodamine B.S. dissolves in water with bluish red colour and yellowish orange fluorescence. It is eliminated very quickly into the glomerulus of a normal winter frog. In the proximal tubules it is partially concentrated by reabsorption of water and partially reabsorbed, staining the protoplasm of the epithelium diffusely. It passes through the lumina of the lower urinary tract in a higher concentration, simultaneously staining their epithelia. Besides this staining from the lumen some time later, a second staining starting from the lymph spaces takes place, simultaneously staining the glomerular epithelia and capillaries, the epithelia of all the tubules and the walls of the blood vessels. The staining of the epithelium of the proximal tubules with rhodamine B.S. starting from the lumen is similar to that with fluorescine, outlasting the period of maximum excretion of the dye and disappearing immediately after the death of the epithelial cells. The staining with rhodamine B.S. starting from the lymph spaces, however, is similar to that with trypaflavine persisting even after death. In the kidney of the winter frog poisoned with phloridzine, the reabsorption of rhodamine B.S. and the quick staining of the epithelium of the proximal tubules do not occur, but later a partial staining of this epithelium takes place from the lymph space.

While in the normal kidney the contents of the complete urinary tract are fluorescent with a yellowish orange colour in the kidney of the frog poisoned with phloridzine, the contents of the lower urinary tract starting from the proximal tubules are surprisingly fluorescent with a very intense bluish green colour. We had not enough evidence yet to explain the nature of this colour change. The alteration of the *p*H cannot be the reason for it, as it is not possible to produce the change of colour by alteration of the *p*H *in vitro*.

The experiments with fluorescine and rhodamine B.S. demonstrate that the reabsorption of substances other than carbohydrates is also affected by phloridzine. Therefore the suggestion of Ellinger [1929] that the glucose present in the phloridzine molecule may interfere with the reabsorption of sugar cannot be correct. The fact that phloridzine affects the reabsorption of fluorescine, too, could be in favour of Lundsgaard's [1933] theory as the fluorescine molecule contains two phenolic OH groups at which a phosphorylation could take place. Since, however, the reabsorption in the proximal tubules is affected in the same manner for rhodamine B.S. which contains, instead of the phenolic OH groups of

fluorescine, two $N(C_2H_5)_2$ groups and which therefore cannot be phosphorylated, Lundsgaard's theory cannot be correct either.

The observations with trypaflavine on the one hand and with fluorescine and rhodamine B.S. on the other hand show (1) that in the kidney poisoned with phloridzine the reabsorption of some substances other than glucose is interrupted, (2) that the staining of the epithelium of the proximal tubules from the lumen does not take place, and (3) that the staining of the epithelium of the proximal tubules from the lymph or the blood capillaries remains unaffected. Therefore phloridzine interferes with the passage of certain substances through the epithelium of the proximal tubules from the lumen into the epithelium. We do not know the mode of this blockade. Perhaps quantitative or qualitative alterations of the electric charge of the membranes might account for it.

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The sugar utilization of hypophysectomized-depancreatized cats. By CHARLES REID

Houssay and co-workers have shown that hypophysectomy clearly attenuates diabetes due to removal of the pancreas. For example, attenuation is shown by the lowered excretion of nitrogen of the fasting hypophysectomized-depancreatized animal as compared with the fasting depancreatized animal. Whether the lowered sugar utilization of the depancreatized animal is improved by hypophysectomy does not appear to be clearly established.

In the following table the results are given of experiments in which cats (*a*) normal, (*b*) depancreatized, (*c*) hypophysectomized-depancreatized, fasted for 48 hours, were given glucose into a vein under chloralose anæsthesia at about 0·5 g. per kg. body weight per hour for 6 hours. The sugar utilization of each preparation was determined by subtracting from the amount given the sugar lost in the urine and the excess sugar in equilibrium with the tissues at the end of the experiment [cp. Lovatt Evans *et al.* 1931]. In some experiments respiratory quotients and liver glycogen formation were also studied, and in others sterile infusions of sugar were done and repeated later in the same animals.

Nature of preparation	Average sugar utilization g./kg./hr.
Normal (8)	0·274 [0·25 –0·310]
Depancreatized (6)	0·195 [0·185–0·198]
Hypophysectomized-depancreatized (8)	0·130 [0·100–0·147]

From the above table it is clear that the sugar utilization of the fasting hypophysectomized-depancreatized cat is much lower than that of the normal fasting animal. Further, it should be borne in mind that Yates *et al.* [1933] have shown that the sugar utilization of the tissues of the depancreatized animal is considerable.

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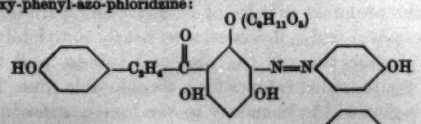
Note on the localization of the glycosuric action of phloridzine.

By P. Ellinger and A. Lambrechts

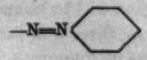
There is a general consensus of opinion that the glycosuric activity of phloridzine is localized in the kidney. But there is still much discussion in which part of the kidney this attack may occur. Cushny suggested that phloridzine may interrupt the reabsorption of glucose in the proximal tubules. Numerous experiments carried out with different methods in order to elucidate this point had contradictory results, but the experiments of Walker & Reisinger showed beyond all doubt that the glomerular elimination of the preliminary urine is not affected at all by phloridzine. In order to clarify this question we examined the behaviour of coloured derivatives of phloridzine on the kidneys of frogs (*Rana esculenta* from Hungary) of the summer and winter type and of rats with the intravital microscope of Ellinger & Hirt.

The following phloridzine derivatives were used:

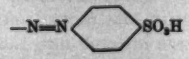
(1) *p*-hydroxy-phenyl-azo-phloridzine:



(2) phenyl-azo-phloridzine:



(3) *p*-sulphonic acid phenyl-azo-phloridzine:



All these substances have colours varying from yellowish red to reddish brown in the solid state as well as in solution. The solutions produce when put between a source of light and a fluorescent substance an intense

brown shadow on the latter. Substance no. 3 can easily be dissolved in water or Ringer's solution. Substances nos. 1 and 2 are nearly insoluble in water but easily soluble in alkali. For the experiments, no. 3 was dissolved in Ringer's solution, while nos. 1 and 2 were dissolved in sodium hydroxide diluted with Ringer's solution and neutralized to such extent with hydrochloric acid as to leave the azo-phloridzines just dissolved.

The kidneys of the frogs narcotized percutaneously with urethane were exposed and observed with the intravital microscope. 1 c.c. of a 1 °/₀₀ fluorescine solution was injected into a leg lymph sac. Soon the lumina of the whole urinary tract from the glomerular capsule to the ureter were filled with the brightly fluorescent dye which stained also the epithelial cells of the proximal tubules. This staining of the epithelium persists even when the elimination of the dye is declining. In a few minutes the excretion of the dye came to its height. Then 2–8 mg. of one of the azo-phloridzines in 2·5–5 °/₀₀ solution were injected into the abdominal vein in the direction opposite to the blood flow.—The azo-phloridzines when injected into the lymph sac or the peritoneal cavity remained where injected. Their injection into the abdominal vein in the direction of the blood flow caused frequently a diastolic stopping of the heart.—A few seconds after the injection, the azo-phloridzines were eliminated into the glomerular capsule and observed as a dark brown shadow lasting for a time dependent on the amount of the injected phloridzine. This glomerular elimination was the same for all three azo-phloridzines used in our experiments. But from this point onwards there was a fundamental difference in the behaviour between the substances nos. 1 and 2 on the one hand, and no. 3 on the other hand. The latter was intensely concentrated by reabsorption of water in the proximal tubules and passed in this high concentration through the lower tubules into the ureter and the bladder. No reabsorption or fixation of the substance could be observed in the proximal tubules or in any other part of the kidney. The substances nos. 1 and 2, however, were nearly completely reabsorbed during their passage through the proximal tubules and fixed in the epithelium of the proximal tubules for a considerable time. They formed there a dark region at the boundary to the lumen, spreading gradually over two-thirds to three-quarters of the epithelial cells, leaving only a narrow bright line of fluorescence bordering the blood capillaries.

The experiments carried out on rats in the same way, where the fluorescine was injected into the peritoneal cavity and the azo-phloridzines into a small side branch of the jugular vein, gave practically the same results.

The three azo-phloridzine compounds had been examined previously on dogs with regard to their glycosuric activity. In these tests only the

substances nos. 1 and 2 had been proved to be active, no. 3 being completely inactive.

Therefore only those phloridzine derivatives produce a glycosuria which are fixed in the epithelial cells of the proximal tubules, and the conclusion may be drawn that the point of attack of phloridzine is localized in the epithelium of the proximal tubules of the kidney. It remains only to discover the conditions under which the azo-derivatives of phloridzine are reabsorbed and fixed in the proximal tubules or are completely excreted in the urine. Our experimental data are not yet sufficient to decide this question conclusively, but the relative solubility in aqueous solutions and lipoids respectively, their dissociation in the aqueous media of the lumen of the urinary tract and the nature and intensity of their electric charge may play an important role in this connexion.

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The formation of acetylcholine by brain tissue. By EDGAR STEDMAN and ELLEN STEDMAN. (*From the Department of Medical Chemistry, University of Edinburgh*)

In an investigation of the mechanism of the biological formation of acetylcholine, Quastel *et al.* [1936] have recently shown that when fresh brain slices are suspended in Ringer's solution and incubated at 37° in the presence of eserine, a substance possessing the properties of a cholin-ester is formed and diffuses into the suspension fluid. During the past two years the present authors, who have also been occupied with this theme, have found that a similar substance is formed when minced brain tissue is ground with a solution of eserine in chloroform and the mixture then incubated at 37° for 1–4 hours. In the absence of chloroform little, if any, of the ester is produced. This preparation appears to be a suitable one for use in determining the actual precursors of acetylcholine, and some provisional experiments in this direction have already been carried out. Before proceeding further with such work, however, it appeared to be desirable to establish with certainty that the substance so produced, the presence of which was recognized by the action of extracts of the incubated material on the usual eserinized leech preparation, was, in fact, acetylcholine. This has now been effected by its chemical identification. Using about forty ox brains, representing some 10 kg. of material, it has been possible, after submitting them to the above treatment, to prepare from them extracts from which acetylcholine has been isolated in the form of its double platinichloride with choline. 60 mg. of this salt, representing 18 mg. of acetylcholine, were obtained. This was identical in crystalline form and melting point with an authentic specimen of the substance. For further identification, the bases recovered from the platinichloride were converted into aurichlorides, from which 20 mg. of

nearly pure acetylcholine aurichloride were obtained. Recrystallization of this material gave 15 mg. of the pure salt, which was identified by comparison with an authentic specimen and by analysis.

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Effects of progesterone and testosterone on *Xenopus* and on its excised ovary. By H. A. Shapiro and H. Zwarenstein. (*From the National Institute for Medical Research, Hampstead*)

It has recently been shown that progesterone [Zwarenstein, 1936], as well as certain related compounds such as testosterone and methyl testosterone [Shapiro, 1936*a*], will cause ovulation in the normal or hypophysectomized *Xenopus*. Frogs caused to ovulate by this means show the hyperæmia of the cloacal labia similar to that seen after injection of gonadotropic extracts of anterior pituitary [Shapiro, 1936*b*] or pregnancy urine [Shapiro & Zwarenstein, 1935]. Attempts have now been made to induce this hyperæmia directly in the ovariectomized frog, but neither testosterone (1 mg. intramuscularly), nor progesterone (1 mg. intramuscularly, or 0·5 mg. intraperitoneally) evoked the response. In addition, œstrone (up to 1 mg.) or œstradiol (up to 1·2 mg.) intramuscularly were ineffective, as were gonadotropic preparations of anterior pituitary and pregnancy urine. It would thus appear that the labial enlargement may be due to some substance, apparently different from known mammalian sex hormones, which is secreted by the amphibian ovary when caused to ovulate.

Coupling with fertilization of eggs, as well as ovulation, were obtained in experiments in which males were injected with saline extract of sheep anterior pituitary and the females with progesterone (1 mg.), testosterone (1 mg.) or methyl testosterone (0·5 mg.).

Ovulation can be induced also in the excised ovary suspended in frog's Ringer solution by the addition of progesterone to the bath [Zwarenstein, 1937], and it is now found that testosterone (1 mg.) and androstenedione (10 mg.) are similarly effective.

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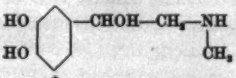
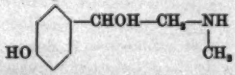
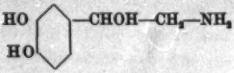
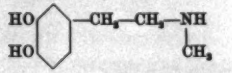
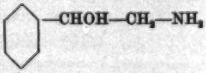
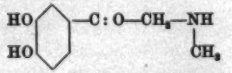
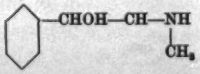
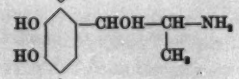
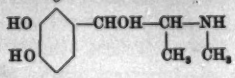
Biological oxidation of adrenaline derivatives. By H. BLASCHKO, D. RICHTER and H. SCHLOSSMANN. (*From the Physiological Laboratory, Cambridge*)

A number of substances structurally related to adrenaline were incubated with extracts from liver tissue (guinea-pig, rat) containing adrenaline oxidase in the presence of 10^{-3} *M* HCN. The results obtained are seen in Table I.

TABLE I

Substance	Structure	Oxygen uptake in liver extracts + 10^{-3} *M* HCN as atoms per molecule of substance	Remarks
Adrenaline (d- and l-)		1	Rate of oxidation d : l approximately 1 : 2
p-Sympatol (d- and l-) (Boehringer)		1	
Arterenol (dl-) (I.G. Farben)		1	Biological inactivation complete after oxidation
Epinine (Burroughs, Wellcome)		1	Oxidation rate faster than that of adrenaline
β-phenyl-β-hydroxy-ethyl-amine (Hoffmann-La Roche)		1	
Adrenalone (I.G. Farben)		>1	Formation of orange-coloured product in 10^{-3} *M* HCN
Ephedrine (d- ψ- and l-) (Fraenkel & Landau)		0	
Corbasil (I.G. Farben)		0	
α-3-4-dihydroxy-phenyl-α-hydroxy-β-methylamino-propane (I.G. Farben)		0	

The following substances reacted in a manner similar to adrenaline: *arterenol*, nor-adrenaline, a substance containing a non-methylated

—NH_2 group, *p-sympatol*, a compound with only one phenolic —OH group, *β-phenyl-β-hydroxy-ethylamine*, a substance without phenolic —OH groups and with a side chain corresponding to that of arterenol, and *epinine*, a substance without the —OH group in the side chain.

If it be assumed that adrenaline oxidase is responsible for the oxidation of these different substances, it follows that the primary point of attack of the enzyme is not at any position in the molecule in which the structure can be varied without affecting the ease of oxidation.

On the other hand *ephedrine* and its two 3-4-dihydroxy- derivatives were not oxidized. The last substance differs from adrenaline only in the additional —CH_3 group in the side chain.

These results show that adrenaline oxidase attacks primarily the side chain of the adrenaline molecule and that the configuration

$$\equiv C—CH_2—N=$$

is essential for the reaction.

The authors wish to acknowledge their indebtedness to Messrs C. H. Boehringer Sohn A.G. and to the I.G. Farbenindustrie for the gift of a number of substances.

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The interpretation of potentials led from the superior cervical ganglion. By J. C. Eccles

When the earthed lead is placed on the superior cervical ganglion and the grid lead on its isolated postganglionic trunk (the polarity of the former being given relative to the latter), a preganglionic volley sets up a sharp spike-like diphasic wave (initially negative), produced by the volley of impulses discharged along the postganglionic fibres, and later slow negative (*N*) and positive (*P*) waves. It has been concluded that a considerable part of the negative spike and almost all of the *N* and *P* waves are produced by the somata of the ganglion cells [Eccles, 1935, 1936].

On the other hand, the following evidence has led Bishop [1936] to suggest that, so far as the recording of action potentials is concerned, the ganglion is equivalent to an inert mass of conducting tissue applied to the postganglionic fibres, which alone produce all the above potentials.

(1) With all positions of the earthed lead on the ganglion the conduction time and form of the spike are practically unaltered. The practically simultaneous arrival of the relatively fast preganglionic impulses at all regions of the ganglia provides, however, a satisfactory explanation. (2) When cocaine is applied to the cut end of the ganglion (postganglionic trunk cut off), the spike is greatly diminished. This would seem to be no more than the "killed-end effect" of peripheral nerve [cf. Eccles, 1935]. (3) The spike recorded from the proximal ganglionic enlargement is smaller than that from the central neck. However, most of the cells in the proximal enlargement send their axons along branches of the ganglion other than the main postganglionic trunk, and so will make no appreciable contribution to the spike with the above leads [Eccles, 1935]. (4) It is stated that no potential is produced by impulses reaching

the synaptic ends of the preganglionic fibres, and on analogy it is assumed that no potential is produced also at the site of origin of impulses discharged along postganglionic fibres, i.e. presumably by the ganglion cells. This experiment receives no confirmation from cats' ganglia, is difficult to reconcile with nerve theory, and in any case the analogy with postganglionic fibres is inadmissible. (5) Finally an inert mass of tissue applied to a nerve is shown to produce virtual leads at its margins, exactly as is suggested for the superior cervical ganglion in its relation to the postganglionic trunk. But such an argument begs the question, for, if the ganglion cells, which form the main bulk of the superior cervical ganglion, themselves produce a spike, then the ganglion will not be an inert mass shunting the spike set up by the postganglionic fibres.

Thus Bishop's suggestion receives no unequivocal experimental support, and in any case he only investigated the spike, asserting that the same considerations must apply to the slow potential waves. On the other hand, there is now much experimental evidence, not discussed by Bishop, which strongly suggests that impulses traverse the somata of ganglion cells, there setting up a spike [cf. Eccles, 1936, p. 353, for details], and finally the following evidence (also not discussed by Bishop) shows conclusively that the greater part of the *N* and *P* waves arises in the ganglion cells.

(1) The *N* and *P* waves are recorded normally when one lead is on the ganglion and the other on the intact postganglionic trunk [Eccles, 1935]. Under such conditions any slow potentials produced by the postganglionic fibres could not be recorded to any appreciable extent. (2) As the earthed lead is moved along the postganglionic trunk away from the ganglion, the *N* and *P* waves show a rapid decrement which cannot be attributed to the "killed-end effect" [Eccles, 1935]. (3) Changes in the excitability of the ganglion cells as tested by preganglionic impulses, i.e. C.E.S. and C.I.S., are closely associated with the respective *N* and *P* waves [Eccles, 1935]. (4) A maximal preganglionic volley always sets up a larger *N* wave than a maximal antidromic volley [Eccles, 1935], hence part at least of the *N* wave must be directly produced in the ganglion cells by preganglionic impulses, and this must also occur for ganglion cells of the "subliminal fringe".

Electrotonic spread along the postganglionic fibres of potentials arising in the ganglion cells is regarded by Bishop as an assumption receiving no support from the behaviour of peripheral nerve. However, the classical experiments on electrotonus of nerve provide an exact parallel, there being even quantitative agreement [Eccles, 1935].

The available evidence thus establishes that the *N* and *P* waves largely arise in the ganglion cells, and suggests a similar origin for part of the spike [Eccles, 1936]. The alternative interpretation suggested by Bishop [1936] is shown to be untenable.

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The action potential of motoneurones.

By J. C. Eccles and J. J. Pritchard

The earthed lead to amplifier and cathode ray oscillograph is placed on the isolated seventh post-thoracic ventral root of the cat, while the grid lead is on the cord just lateral to the entry of this root. An antidromic volley (set up at a more distal part of the seventh ventral root) produces an initial large diphasic spike. The negative phase of the spike (polarity of spinal cord is always stated relative to root) continues on to a slow negative wave which may even show a rising phase for 5 msec. or more before declining to the later positive wave which reaches a maximum usually at 20–40 msec., and eventually disappears at 70–200 msec. These waves cannot be after-potentials set up by the antidromic volley in the ventral root fibres, for the leads are symmetrical with regard to these fibres, and in any case they are too large, being as much as 1 mV. Moreover, since they decrement rapidly as the grid and earthed leads are together moved peripherally from the cord along the ventral root, being halved in about every 3 mm., they must spread electrotonically from the spinal cord [cf. Barron & Matthews, 1936]. In many experiments one or more small diphasic spike potentials (initially negative) are superimposed on the negative wave. Their polarity and shape show that they are due to imperfectly synchronized impulses discharged spontaneously from some motoneurones. As the size of the antidromic volley is diminished, the negative and positive waves and the spontaneous discharges all diminish in parallel with the initial spike, i.e. in setting up these three responses in the spinal cord there is no detectable interaction between the individual antidromic impulses. Presumably each antidromic impulse sets up the negative and positive waves only in the soma of its own motoneurone. In ganglion cells antidromic impulses set up

similar potential waves which are composed of overlapping *N* and *P* waves [Eccles, 1936], and presumably in motoneurones the recorded potential is similarly composed, the only differences arising on account of the shorter duration of these waves, the relatively larger *N* wave, and the spontaneous discharge. In the interaction of the *N* and *P* waves set up by two maximal antidromic volleys there is also a close resemblance with ganglion cells. The reflex response of motoneurones to a testing dorsal root volley shows that their excitability is increased during the *N* wave and diminished during the *P* wave, a correlation also indicated by the spontaneous discharge during the *N* wave, and the diminution or abolition of this discharge when an antidromic volley is set up during the *P* wave of a previous volley. These *N* and *P* waves and the associated changes in excitability have been already discussed in relation to the problems of reflex action [cf. Eccles, 1936, pp. 386–97].

With similar leads a very small dorsal root volley sets up a slow negative wave with latent period about 1 msec. and a rising phase about 10 msec. A larger volley sets up a spike usually with a latent period about 4 msec. (the central reflex time), and with a still larger volley there is usually an earlier spike, often very large, after a central reflex time of only 0·7–1 msec. Ventral root leads show that both these large spikes are due to approximately synchronized motoneurone discharges. On the other hand, the negative wave suffers a decremental transmission along the ventral root exactly similar to the antidromic slow waves; hence presumably it also spreads electrotonically from the motoneurones. Facilitation of the response to a dorsal root volley may be observed if it is set up shortly after a previous volley, and the central reflex time of the second spike may be shortened to equal that of the first. At longer intervals there is frequently a prolonged depression which seems to occur in internuncial pathways, for the slow negative wave is affected as well as the spikes, and this does not occur for the depression associated with the *P* wave set up by an antidromic volley. The destination of the impulses of the first spike is unknown, for, with the flexor reflex response of tibialis anticus [Eccles & Sherrington, 1931], the central reflex time and its diminution by facilitation correspond to the second spike. The central reflex time for the first spike and for the facilitated second spike approximately equals the synaptic delay for a single synapse elsewhere in the central nervous system [cf. Eccles, 1936, p. 388], hence presumably impulses in the dorsal root fibres directly set up a discharge from the motoneurones. The synaptic delay is so short that this discharge must occur before the slow negative wave, which presumably is associated with

that increased excitability of neurones due to C.E.S. Hence it seems that this discharge is not due to C.E.S., but to the rapid initial excitatory process which is called the detonator response [cf. Eccles, 1936].

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Responses of denervated smooth muscle.

By J. C. Eccles and J. W. Magladery

In one of their four experiments Monnier & Bacq [1935] found that the denervated (20–30 days) nictitating membrane of the cat contracted in response to direct electrical stimulation. After injection of 933 F direct stimulation elicited a response in all experiments. In three experiments Rosenblueth *et al.* [1936] were unable to elicit any contraction by direct stimulation even after 933 F, and they suggested that Monnier & Bacq were not investigating true contractions of the nictitating membrane. Their criticisms have been answered by Monnier [1936].

We have denervated the nictitating membrane in fourteen experiments by removing the superior cervical ganglion, the completeness of this removal being checked by histological examination. Condenser discharges were employed to stimulate the muscle, and its contraction was recorded by a frictionless isometric optical myograph giving a deflexion up to 6 cm. for 1 g. tension. In our three experiments, after only 7 days' degeneration, the smooth muscle was as easily excitable as on the normal side and the responses were large and irregular, but no accurate investigation was possible as there were also irregular spontaneous contractions. In one experiment the degeneration period was 48 days, and in the remaining ten experiments it varied from 9 to 22 days. In all these experiments a small contraction of the smooth muscle was evoked by direct stimulation. There was a definite threshold, and with increase in the stimulus the response reached a maximum. The contraction began about 0·2 sec. after the stimulus, reached its summit (usually 0·1–0·2 g. tension) about 2 sec. later and then declined steadily, thus

resembling a rather slow twitch response of normal smooth muscle when this is uncomplicated by rhythmic waves. The threshold stimulus was not very intense, e.g. 18 volts declining to half in 35 msec. A second maximal stimulus failed to elicit any response unless it followed the first by more than about 0·2 sec., but the absolutely refractory period may be shorter than this value, as very strong stimuli were not used. Injections of 933 F usually increased the size of the contraction, the most spectacular results being obtained after 9–16 days' degeneration.

Small intravenous doses of adrenaline, e.g. 5γ, evoke contractions of normal muscle which, as shown by the action potentials, seem to be almost entirely due to impulses rhythmically generated in the smooth muscle fibres. With larger doses, e.g. 25γ, a contracture of the muscle with cessation of the action potentials supervenes on the initial phase of contraction set up by the rhythmic discharge [Eccles & Magladery, 1937]. Adrenaline seems to produce almost entirely this contracture response in denervated muscles, for the action potentials produced by the rhythmic impulses are always much smaller, and persist for a shorter time than with the normal membrane, though the mechanical response is larger and longer. A similar effect of denervation has been observed by Brown [1936] in striated muscles. Normally acetylcholine evokes contractions on account of the impulses which it sets up in the striated muscle fibres, while in a denervated muscle a large dose of acetylcholine produces a short burst of impulses followed by a prolonged contracture during which no impulses are set up. Hence smooth muscle only differs from striated muscle in its greater propensity to give contractures both in the normal and denervated state.

Since denervation thus makes it more difficult for adrenaline to set up impulses in smooth muscle fibres, it seems probable that normal smooth muscle would be electrically more easily excitable by direct stimulation than the denervated muscle, and would also give larger responses.

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The spinal pathways of the pupillo-dilator reflex.

By A. A. Harper and B. A. McSwiney

The pathways in the spinal cord of visceral afferent fibres in the splanchnic nerves and of somatic afferent fibres in the intercostal nerves have been investigated, dilatation of the pupil in cats anæsthetized with chloralose being taken as an index of afferent fibres.

Both visceral afferent and somatic afferent fibres pass up the spinal cord ipsilaterally and contralaterally in the lateral columns of white matter.

Those fibres, whether visceral or somatic, which cross the spinal cord, do so in the segment above that by which they enter the cord.

Peripheral excitation and inhibition of a sense organ.

By A. Sand

A technique has been developed for perfusing a portion of the hyomandibular lateral line canal of the elasmobranch fish, *Raja*. The influence of the movement of fluid along the canal upon the activity of a single end organ has been recorded with a Matthews oscillograph.

In a state of rest the end organ possesses a spontaneous rhythmicity and discharges afferent impulses at the rate of 8 or 9 per sec.

Fig. 1 shows successive portions of the record of a continuous experiment in which the canal was perfused first in a headward direction, then in a tailward direction at a constant rate of flow, equivalent to a displacement of the fluid in the canal at an approximate rate of 12 mm. per sec.

Record A. Beginning of 10 sec. headward perfusion. The rhythm is accelerated and adaptation is very slow.

Record B. End of this perfusion. When the tap is turned off the discharge stops completely for 28 sec.

Record C. 28 sec. later. No perfusion. The spontaneous rhythm begins again.

Record D. No perfusion. The spontaneous rhythm 60 sec. after the end of perfusion in B.

Record E. Beginning of 10 sec. of tailward perfusion. No impulses for 2 sec., then a slow rhythm at 3 and 4 per sec.

Record F. End of this perfusion. The spontaneous rhythm returns.

Record G. No perfusion. 9–12 sec. after the end of perfusion in F. The frequency is higher than in D.

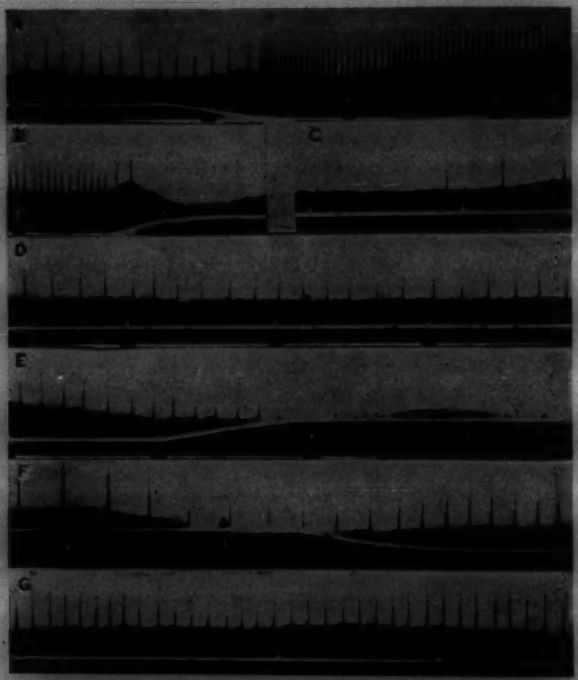

Fig. 1. Oscillographic records, reading left to right, of the activity of a single end organ of the lateral line of *Raja* in response to perfusion of the hyomandibular canal. The white line is the perfusion signal. Its downward displacement signals the beginning of headward perfusion, its upward displacement signals tailward perfusion. Time signal in sec.

The receptor is sensitive to a movement of fluid along the canal at a rate of 0–16 mm. per sec. approximately, and over this range the relation between frequency and rate of headward (excitatory) flow is logarithmic.

The behaviour of this receptor is identical with that of the ampulla of the horizontal semicircular canal of the dogfish [Löwenstein & Sand, 1936].

Whether the action of a tailward flow in abolishing or reducing the spontaneous rhythm can be regarded as true inhibition of the receptor appears to depend largely upon definition, but the processes here recorded bear a sufficiently striking resemblance to the phenomena of excitation and inhibition of a motoneurone in the central nervous system to invite speculation as to their ultimate identity. Is the spontaneous rhythmicity of resting acustico-lateral receptors a manifestation of a peripheral excitatory state?

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Further observations on endocrine interactions in the menstrual cycle. By S. Zuckerman

Experiments reported previously [Zuckerman, 1936*a*] show (*a*) that uterine bleeding which would normally occur in ovariectomized rhesus monkeys injected daily with a threshold dose of œstrin can be inhibited by the simultaneous administration of progestin, and (*b*) that uterine bleeding which would normally follow a course of progestin injections may be inhibited if an amount of œstrin that is above the threshold level is given daily both during and after the course of progestin injections. Further observations suggest that the occurrence of uterine bleeding during courses of injections in which œstrin and progestin are given simultaneously is dependent on the outcome of the same kind of hormonal antagonism as decides in the case of the vagina [Allen & Meyer, 1935], or of the prostate [Zuckerman & Parkes, 1936; Zuckerman, 1936*b*] whether or not œstrogenic effects will prevail. The following are some of the experiments on which this conclusion rests.

Progestin is without effect on the uterus of a spayed monkey (or non-primate mammal) unless the animal has been previously injected with œstrin. Thus fourteen successive daily injections of 2 rabbit units of progestin into an ovariectomized rhesus monkey were not succeeded by uterine bleeding (186. 1). On the other hand, bleeding did follow 4 days after a similar course of injections, on the 6th day of which 2000 I.U. of œstrone were also given (186. 2). In a third experiment

(70. 7) 2 R.U. of progestin were given to a spayed monkey daily for 12 days after a preliminary 14 day period in which only œstrone was injected (2000 I.U. daily). At the end of the period of progestin injections the animal was given 500 I.U. of œstrone daily. Uterine bleeding set in on the 29th day of the experiment, 3 days after the cessation of progestin injections, and in spite of the fact that the animal was being injected with an amount of œstrone well above the threshold level necessary to maintain the endometrium in an interval condition. A corresponding finding has been made by Engle *et al.* [1935], and it suggests that once the stimulus that has produced full progestational changes in the uterus is withdrawn, œstrone cannot prevent the consequent and usual breakdown of the endometrium.

Uterine bleeding also occurred on the 29th day of an experiment which was identical with the third (70. 7) except for the daily administration between the 15th and 26th days of 50 I.U. of œstrone as well as 2 R.U. of progestin. On the other hand, it did not occur until after the cessation of œstrone injections (500 I.U. daily) on the 40th day of a similarly planned experiment (45. 3) in which 500 I.U. of œstrone were given each day during the period progestin was being administered. Presumably the progestin given in this experiment was in some way neutralized by the œstrone that was simultaneously injected, the œstrone also maintaining the uterus in the phase of growth begun during the first period of œstrone injections.

An adequate amount of progestin will have the opposite effect, and will successfully counteract the influence of œstrin. Thus a spayed rhesus (180. 2) was first injected daily for 14 days with œstrone alone (3000 I.U. daily). Between the 15th and 26th days it was given 500 I.U. of œstrone, together with 10 mg. of progesterone daily. On the 27th day the progesterone injections were stopped, the daily administration of 500 I.U. of œstrone being continued. Uterine bleeding set in on the 30th day in spite of the fact that the animal had never bled during previous courses of injection of similar amounts of œstrone. Histological study showed that the uterus was in an interval and not in a progestational condition. Presumably the progesterone had reduced the effective value of the œstrone given between the 15th and 26th days to a subthreshold level, while its interaction with œstrone rendered it ineffective from the point of view of the production of progestational changes in the endometrium. The fact that the raising of the œstrone level after the 27th day did not inhibit bleeding may be related to the finding that the endometrial processes leading to uterine bleeding are irreversible in

their later stages [Zuckerman, 1936c]. The same result was obtained in four similarly planned experiments in which the ratio of progesterone to œstrone was correspondingly high. Further experiments show that the occurrence or non-occurrence of uterine bleeding during the course of injections in which progestin is given daily, together with an amount of œstrone that would be insufficient to prevent uterine bleeding, is also dependent on the quantitative ratio of the two hormones to each other.

These results are in line with those obtained by Leonard *et al.* [1930] and by Robson [1936] in studies of the effects of progesterone on the structure and reactivity of the rabbit uterus.

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Concentration of the blood in relation to the menstrual cycle in monkeys. By A. N. Guthkelch and S. Zuckerman

Swelling of the sexual skin of pig-tailed macaques during the menstrual cycle can be related to fluctuations in the daily water balance [Krohn & Zuckerman, 1937]. It now appears that cyclical variations also occur in the concentration of red blood cells. Estimations of the number of erythrocytes were made twice weekly during two cycles of the animal that was the subject of the previous study. The number of red cells is lowest during menstruation (between 4 and 4½ millions). It rises rapidly and reaches a maximum (about 5¾ millions) during the time of full sexual-skin swelling. With the mid-cycle onset of sexual-skin subsidence, the number of cells begins to fall, until in the immediate pre-menstrual phase it is again in the neighbourhood of 4½ million.

The main cyclical change in the sexual skin of rhesus macaques, an allied species of monkey, is one of coloration, such swelling as normally occurs being very slight. Observations carried out during five menstrual

cycles show that this species undergoes fluctuations in the number of red cells similar to those that occur in the pig-tailed monkey. Further observations on ovariectomized rhesus monkeys receiving injections of œstrone and œstrone benzoate suggest that the increase in red cells during the earlier half (follicular phase) of the normal cycle results either directly or indirectly from the action of œstrin. Observations were made during courses of injections on fifteen animals. In four experiments estimations were made twice weekly during both the period of injections (14 days) and the latent interval (8–18 days) which intervened between the cessation of injections and the onset of "œstrin-withdrawal" uterine bleeding. In the remaining eleven experiments estimations were made only at critical points during the whole experimental period. With the exception of two animals, which gave no significant results, and one in which the results were somewhat conflicting, all the animals showed an increase (usually more than 1 million) in the number of red cells during the course of injections, with a subsequent fall (of about an equal amount and as a rule to a level of about 4½ million) during the latent period preceding uterine bleeding. The available data do not reveal any correlation between the magnitude of change in the red-cell count and the amount of hormone administered.

Although the red cells of rhesus monkeys fluctuate in number according to the phases of the menstrual cycle, the daily water balance of these animals does not vary in relation to the cycle in as obvious a way as it does in the pig-tailed monkey. Thus cyclical fluctuations in body weight were not detected in three normal rhesus monkeys, each of which was observed through two menstrual cycles, while cyclical variations in the amount of urine excreted, such as are usual in the pig-tailed monkey, could be detected only in one.

The normal course of events in the pig-tailed monkey suggests that less urine should be excreted during than after phases of œstrogenic activity. Observations were therefore made on three ovariectomized rhesus monkeys during eight courses of œstrone injections. As in the case of normal animals, wide daily fluctuations in the amount of urine excreted rendered the analysis of the data difficult, and in only three of the experiments did the observations apparently accord with expectation.

The available data show clearly that water metabolism in the rhesus monkey is not affected by the menstrual cycle to the same extent that it is in the pig-tailed macaque. Moreover if cyclical variation in the red-cell count is an index of cyclical changes in the concentration of the blood, it is possible that such fluid as is sometimes deposited in the sexual

skin of the rhesus during its phases of activity is derived in the main from the blood itself, and not from external sources, as in the pig-tailed monkey. If this inference is correct, it also follows that the blood volume is similarly affected in the pig-tailed monkey, and that the cyclical retention of extraneous water represents a secondary mechanism related to the more pronounced swelling shown by this species.

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The metabolic response to injury. By D. P. Cuthbertson and J. S. Robertson

Previous observations on the human subject had shown that in the days following a severe accidental injury, such as the fracture of one of the long bones or a dislocation of a joint, etc., a marked loss of N, S, P and K but not Na frequently occurred in the urine [Cuthbertson, 1930, 1932, 1936]. A creatinuria paralleled these changes. The loss of body substance was in excess of that produced by simple non-use [Cuthbertson, 1929] and was apparently more general than local. Satisfactory proof of this latter point could not be obtained by clinical observation.

Rats (180 days old) were placed on a constant basal diet just sufficient to maintain weight and N equilibrium, and after stabilization the left femur was fractured by open operation under ether anæsthesia. No splinting was used, and the animals were able to move about remarkably freely within a few hours and appetite did not fail (one exception).

A definite increase in protein catabolism occurred and the maximum N excretion was noted on the 3rd–4th day following the injury. Basal conditions were reached in about 9 days. The animals were killed generally when the N excretion had reached the basal value. Comparative weighings, after skinning, of the symmetrically severed lower limbs with half pelvis, and the knee extensor group of muscles indicated that the loss of weight by this group of muscles represented rather less than half the loss in weight of the injured limb, and that the loss of weight by the latter could only account for a fraction of the total loss of N, presuming

the loss of weight to be due to a loss of muscle substance. Investigations in progress show that there is a marked loss of K but not Na contemporaneous with this N loss, and that this also cannot be accounted by the local and adjacent tissue loss. It would appear that the loss of N, etc., which occurs as the result of injury has its origin in a general catabolism of protein, and the loss of K strongly suggests that the actual cells may lose their substance.

The addition of extra carbohydrate to the basal diet on the days following the injury caused a definite diminution in the N and, to less extent, K excretions. It also prevented the loss in body weight without, however, apparently altering the loss of muscle tissue by the extensor group and by the affected limb.

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The effect of cyanide on the retractor of the byssus of *Mytilus edulis*. By INDERJIT SINGH. (*From the Physiological Laboratory, Cambridge*)

Cyanide induces a powerful contraction of the retractor of the byssus of *Mytilus edulis*; the maximum is reached in a few minutes and is followed by a decline. The sensitivity of the muscles varies with individual muscles and with season. Sometimes they respond to concentrations of 1 in 100,000; at other times they fail to respond to concentrations of 1 in 400.

If a muscle is immersed for an hour or more in a solution of cyanide, at a concentration which is insufficient to produce a contracture, it still responds with a contracture to electrical stimulation (A.C. and D.C.), to barium or potassium, or to sudden stretch, or release from a stretch. In some experiments muscles responded to all these forms of stimulation in concentrations of 1 in 500. If the muscle is immersed in calcium-free *Mytilus* saline for an hour, and then in isotonic sodium citrate for 15 min., even replacement of the whole of the sodium chloride solution in the bath by isotonic sodium cyanide fails to produce a contracture. After

removal of the calcium and treatment with sodium citrate, most forms of stimulation become ineffective, but the muscle may still contract when stimulated by means of a direct current. Such muscles are still capable of responding to stimulation with a direct current even when the sodium chloride in the solution is replaced with isotonic sodium cyanide (0·564 *M*).

Experimental "chronic" lesions in the central nervous system of the sheep's fœtus. By J. Barcroft and D. H. Barron

In a former communication [Barcroft & Barron, 1936] it was shown that (1) a sheep's fœtus of 42 days showed rhythmic movements of a generalized type; (2) by 52 days these have individuated along two lines, (*a*) a rhythm of a respiratory type, and (*b*) somatic movements, the two being linked; (3) by 60 days the movements have become relatively difficult to evoke.

Our present object is the study of this damping of the "respiratory" and somatic movements.

Our method has been to institute a comparison between normal fœtuses and those in which the C.N.S. has been transected at certain levels. The transection of the cord or bulb may be carried out without affecting the normal growth of the fœtus. The transection has usually been done a week to 10 days before the ultimate investigation of the fœtal movements, the latter operation taking place usually between the 70th and 75th day of fœtal life.

As the normal 70 day fœtus deteriorates when exposed, these stages may be recognized in which successively:

(1) The fœtus is very inert: if movement takes place it is of a drawn-out character and does not initiate a respiratory rhythm. Pinching the umbilical cord initiates a respiratory rhythm but not general muscular movements.

(2) The fœtus is more active, the muscular movements are less drawn out and initiate a respiratory rhythm. Pinching the cord induces muscular movements of a kicking type.

(3) Muscular movements may be elicited reflexly; they are rapid and jerky, e.g. kicking of the limbs—no respiratory movements.

In general the body of the spinal fœtus conforms to and exaggerates stage 3 of the normal fœtus; its head to stage 1 or 2. The leg movements are rapid and violent. There are no respiratory movements except such

as depend upon cranial nerves (head and throat). Rhythmic movements more akin to swallowing also occur. The mouth responds to stimulation either by opening or by a deliberate chewing rhythm.

Section below mid-brain but above pons, the fœtus is very active, but perhaps less so than the spinal fœtus. Leg movements are jerky, respiratory movements almost continuous, occasional efforts to orient head.

Section through mid-brain, relation to red nucleus doubtful: movements of legs jerky, but occasional sustained movements; no efforts to orientate the head. Respiratory movements present.

Section above mid-brain, sustained leg movements at first, similar to those in normal fœtus; at a later stage the jerky leg movements appear.

The above phenomena are shown on a film.

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